Mammary Sensitivity to Protein and Energy Intake During Lactation in Rats

Mark George Goodwill

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Declaration

I declare that this thesis has been composed by myself. The experimental work and analyses were carried out by myself, with the assistance of other people as indicated in the acknowledgments. The work in this thesis has not been submitted for any other degree or qualification.

M. G. Goodwill
September 1996.

I lift up my eyes to the hills -
where does my help come from?
My help comes from the Lord,
the Maker of heaven and earth.

Psalm 121:1-2

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ABSTRACT

The importance of dietary protein and energy intake to the development and activity of the mammary gland was investigated in lactating rats. Four rat trials were undertaken. The first examined the influence of protein undernutrition and re-alimentation on mammary gland size and secretory cell activity. The results showed that rats offered a protein restricted diet during lactation suffer mammary underdevelopment, but this may be rapidly reversed by re-alimentation with a diet of high protein concentration. However, lactating rats offered a lower protein concentration diet significantly reduce voluntary food intake, which poses a dilemma of interpretation as it is not possible to definitively ascribe the mammary underdevelopment to a protein or energy deficiency.

The reduction in food intake shown by these rats also led to a significant loss of body weight due to mobilisation of body tissue. It has previously been proposed that mobilisation of muscle carnosine and haemoglobin, both of which contain relatively high levels of histidine, resulted in elevated levels of plasma histidine. This is converted in the brain to the neurotransmitter histamine which acts on the hypothalamus suppressing food intake.

The second trial investigated the role of the histamine receptor antagonist, cyproheptadine on the voluntary food intake of low protein concentration diets offered to both lactating or young, growing rats. This work indicated that lactating rats offered a protein deficient diet increased their voluntary food intake and also lactational ability until day 8 of lactation, when injected intraperitoneally with cyproheptadine, after which intake dropped. However, the drug had no significant effect on the variables measured in the growing animals.

Since cyproheptadine was only effective in lactating rats over the short term, trial 3 was designed to investigate whether the mammary gland's response to protein re-alimentation, seen in trial 1 was due to an increase in the intake of dietary protein, energy or both. This experiment aimed to differentiate the effects of dietary protein

and energy intake on mammary development and milk composition by rationing dietary protein and energy allowances separately. In addition, the ability of the maternal body reserves, both protein and lipid, to supplement dietary inadequacies was investigated. The results concluded that increasing the supply of dietary energy, but not protein induced mammary cell proliferation and increased secretory cell activity, while increased intakes of both energy and crude protein improved lactational performance. However, milk composition was largely unaffected by dietary treatment. Maternal lipid reserves were significantly reduced between day 1 and 10 of lactation in all dietary treatment groups. However crude protein reserves were only catabolised following severe protein restriction.

The rapid increases in mammary cell mass observed during lactation in trials 1 and 3 must have occurred through either an increase in cell division, a decrease in cell death or a combination of both. Trial 4 aimed to develop a method suitable for quantifying rates of mammary cell division *in vitro* using [^{3}H]-thymidine incorporation in explants removed from the mammary glands of rats. A method was developed and it was concluded that the rapid increase in secretory cell mass which occured on re-alimentation with a diet of high protein to energy ratio was a result of a high rate of cell proliferation and a decreased rate of cell death.

TABLE OF CONTENTS

ABBREVIATIONS

ATPase	- Adenosine Triphosphatase
BHT	- Butylated Hydroxy Toluene
CHO	- Carbohydrate
CP	- Crude Protein
DM	- Dry matter
DNA	- Deoxyribonucleic Acid
dsDNA	- Double Stranded Deoxyribonucleic Acid
d.p.	- Decimal Place
D-PBS	- Dulbecco's Phosphate Buffered Saline
dpm	- Disintegrations per minute
ε	- Absorption Coefficient
ECF	- Extra Cellular Fluid
ECM	- Extra Cellular Matrix
EDTA	- Ethylene Diamine Tetracetic Acid
FBS	- Foetal Bovine Serum
Gal-DH	- β-Galactose Dehydrogenase Enzyme
GE	- Gross Energy
HEPES	- N-[2-Hydroxyethyl]piperazine-N'-[2-ethansulphonic acid]
IGF	- Insulin-Like Growth Factor
i.u.	- International Unit
M199	- Medium 199
MEM	- Minimum Essential Medium
MW	- Molecular Weight
NAD^+	- Nicotinamide Adenine Dinucleotide (oxidised)
NADH	- Nicotinamide Adenine Dinucleotide (reduced)
RNA	- Ribonucleic Acid
RUDP	- Rumen Undegradable Protein
SED	- Standard Error of the Deviation
SEM	- Standard Error of the Mean
TCA	- Trichloroacetic Acid
Tris	- Tris(Hydroxymethyl) Aminomethane

CHAPTER 1

INTRODUCTION AND REVIEW OF LITERATURE

1.1 INTRODUCTION

Lactation is a physiological process characterised by the secretion of large quantities of protein, carbohydrate, lipid and other nutrients from a specialised cutaneous organ, the mammary gland. The possession of mammary glands and the ability to secrete milk are common to all female mammals and the sole characteristic distinguishing them from other vertebrates. Milk is a complex secretory fluid containing lipids in emulsion, proteins in colloidal dispersion and various organic and inorganic constituents in aqueous solution (Oftedal, 1984). Milk alone is sufficient to support the development of the newborn through the critical stages of postnatal growth.

The major milk components are synthesised within the gland from precursors derived from the vascular system. Although qualitatively, milk composition is similar between species, quantitatively there is considerable variation (see Davis *et al.*, 1983; Jenness, 1985). Humans, with a relatively long natural lactation period have milk with a low fat and protein concentration (Oftedal,1984). However, animals such as the hooded seal whose lactation period is only four days, provide a very concentrated milk to ensure sufficient pup development to allow the mother to return to sea in order to feed (Oftedal *et al.*,1993).

The nutritional benefits of human milk are well documented (Jelliffe and Jelliffe, 1978) and are of special significance for the health of infants in developing countries (Perez-Escamilla *et al.*, 1995). Thus, it is important to identify those factors that influence a woman's ability to lactate successfully. One common factor is nutrition which has received little scientific attention when compared to the myriad of literature concerning the development and regulation of the mammary gland. An adequate supply of nutrients has been shown to be critical for the maintenance of a good growth rate after weaning and during infancy and breast milk is the best source of these nutrients (Alleyne *et al.*, 1977). A satisfactory supply of milk over a long suckling period depends on the mother's nutritional status and, in developing countries where protein-energy malnutrition is prevalent this is not always possible. However, if our understanding of the links between nutrition and lactational

performance are improved, there may be critical times during lactation when targetted nutritional support would be beneficial.

A further important stimulus for the study of mammary growth is the desire to understand the loss of control involved in the genesis of mammary cancer. Breast cancer affected 180 000 women and killed 46 000 in the United States during 1994 (Medina, 1996). A knowledge of the regulatory processes could help unravel the current uncertainties concerning the links between nutrition and gene expression.

Much of the recent research concerned with lactation has been conducted at the cellular level, however, the effective synthesis of milk is a process that involves metabolic adaptations in the whole animal. The modern reductionist approach, although undeniably valuable, by its very nature does not view the animal as a whole. Lactational performance, after all, is the important parameter for milk production in dairy species and also for the efficient production of meat from animals selected for an ever increasing postnatal growth rate. The UK pig industry is amongst the most technologically developed of the animal industries and it still clearly illustrates the need for a better, holistic understanding of the lactation process. Pre-weaning mortality is as high today as it was 20 years ago (Varley, 1995) and of these deaths, 42 % have been directly attributed to malnutrition resulting from the sow's poor lactational performance (Dyck and Swierstra, 1987).

Throughout the world, milk yields per dairy cow have been increasing (Grant, 1991); yields in the UK have increased by 30 % in the last 20 years (UK Dairy Facts and Figures, 1994). Increases in yield have been achieved through a combination of breeding, nutrition and management with great advances been made in all three areas. Conventional theories of milk production suggest that the volume and composition of milk depends upon the quantity of food offered to the dam, *i.e.* milk production is a nutrient controlled response. It is now recognised that this view is far too simplistic and the quality of the diet must be considered, the most important aspects being the supply of metabolisable energy and protein. Modelling the supply of energy and protein relative to the animal's demand is a potentially cheap and rapid method of improving milk production.

In this thesis, each experimental chapter is preceded by a specific review of literature pertinent to the experiment, therefore the following review is a general introduction to this thesis.

1.2 MAMMARY DEVELOPMENT

The fully developed mammary gland is composed of two primary components: the parenchyma, which, in common with all exocrine glands, is composed of secretory and ductal tissue; and the adipose stroma, which provides a substrate within which the parenchyma develops and functions. Parenchyma consists of specialised epithelial cells (secretory cells), myoepithelial cells and ductal cells.

The basic structure of the mammary gland is laid down during foetal development, after which development is a progressive event, with important phases at puberty and pregnancy. The mammary gland then undergoes a cycle of proliferation, differentiation and involution on each successful mating. Since mammary regression is sometimes incomplete between successive lactations (for instance modern management of the commercial dairy cow), the starting point for each cycle may be prior to the end of the previous cycle.

1.2.1 Methodology

Mammary gland development has been described in many ways by various techniques, each of which has both advantages and limitations. In order to follow accurately the development of the mammary gland, it is necessary to determine both its size and internal composition. Quantitative changes in mammary gland morphology, for example weight, circumference or volume may be easily measured post mortem and also on live animals in species with large pendulous glands. Unfortunately, these methods tend to show large variations between animals due to different fat and connective tissue contents (Rattray *et al.* 1974; Munford, 1964; Fowler *et al.*, 1990). However, due to its non-invasive nature, this approach can be useful for following the course of mammary development in individual animals, which

have a high ratio of functional (parenchymal) to non-functional (stromal) cells in the gland, for example the goat (Linzell, 1966). Data for mammary gross size are more informative when used in conjunction with the analysis of internal composition.

Composition can be determined biochemically and / or histologically. Determination of internal nature is invariably invasive; either tissue is removed post mortem or biopsy samples are removed surgically. Laboratory animals are relatively cheap and available in large numbers, so it is possible to sacrifice groups of animals at appropriate stages and remove the whole gland to determine its biochemical composition. As animals of one genetic strain can be used and a mean group value calculated for each variable, variation between animals is low and may be assumed to be random. However, this method is not viable, on both economic and ethical grounds for large domestic species or human subjects. Biopsies allow samples to be removed from the same animal over a period of time; however the reliability of the results depends on the samples being representative of the whole gland. This method has been successfully used for the lactating cow (Hibbit, 1964; Knight *et al.*, 1992b) and also the goat (Knight and Peaker, 1984a); however, non-lactating mammary tissue is not homogenous and therefore this method is not valid for the analysis of non-lactating glands (Knight and Peaker, 1984b).

It is also possible to remove glands surgically at different time points for complete analysis, although this method is clearly restricted by the number of glands present and also the removal of glands may stimulate compensatory growth in the remaining glands (Knight and Peaker, 1982c). Although it is possible to carry out useful quantitative histological studies on mammary tissue, the soft tissue makes it hard to study and it can be very time consuming. Biochemical analysis, on the other hand is relatively quick and accurate. As DNA is located almost entirely in the nucleus and its mass is constant within the diploid mammary cell (Griffith and Turner, 1957; Tucker and Reese, 1962) except during the S-phase and mitosis, total tissue DNA (DNA_t) may be used as an accurate indicator of cell number and therefore development (Naito, 1958; Tucker, 1987). For this to be a true measurement of change in the secretory cell mass, as opposed to the stromal mass, the assumption that the adipose and connective tissue cell population remains constant must be made.

Paape and Sinha (1971) showed that the stromal cell population of the mammary gland is largely determined before first conception and therefore any change in DNA_t noted after this time may be ascribed to the epithelial cell population. Consequently this is a valuable method for measuring glandular development after first conception. Since RNA is intimately related to the biosynthesis of protein, this nucleic acid may be used as a good indication of the cells synthetic potential (Winick and Noble, 1965). However, mammary epithelial cells synthesise both cellular and milk proteins and using this method alone, it is not possible to differentiate between these two pathways.

Although these indices give an accurate indication of the stage of development of the gland, they are static measurements. It is also possible to measure the gland's actual growth rate at any point in time. Colchicine, a plant alkaloid, has been widely used to arrest mitosis in metaphase and hence determine the proportion of cells engaged in proliferation histologically (Reece and Warbiton, 1953; Dilley, 1971; Prasad, 1976). Alternatively, mitotic indices may be determined by incorporation of the DNA base, thymidine that has been radiolabeled with tritium and visualised autoradiographically (Borst and Mahoney, 1980) or quantified by liquid scintillation (Knight and Peaker, 1982a; Woodward *et al.*, 1993). However, it should be remembered that these measurements are only useful if the size of the cell population is also known. Borst and Mahoney (1980) have reported diurnal variations in mammary gland DNA synthesis in the mouse, and Bresciani (1971) noted that endocrine status affected rates of thymidine incorporation. Therefore, comparisons of data should be made with caution, unless the conditions under which measurements were made are similar.

The above methods quantify secretory cell number, however it must be remembered that only differentiated cells are able to synthesise and secrete milk. Milk yield is a function of both the number of differentiated cells available to synthesise milk and also the activity of these cells. Measurements of mammary development must, therefore, also include measurements of cellular activity. Studies quantifying cellular activity have concentrated on the activity of key mammary enzymes, for example acetyl-CoA carboxylase, fatty acid synthetase, lactose synthetase and

galactosyltransferase (Baldwin, 1966; Wilde and Kuhn, 1979; Mansaray and Grimble, 1983 and 1984; Wilde *et al.*, 1986; Shipman *et al.*, 1987; Fowler *et al.*, 1991a). The activities of these enzymes are assayed *in vitro* and as a result, the activities obtained may not be a true representation of the *in vivo* situation; when similar tissue is assayed under identical conditions, the relative differences are a useful indication of cellular activity. The rates of lactose production observed *in vitro* by both Wilde and Kuhn, (1979) and Mansaray and Grimble, (1984) agree extremely closely with the observed milk yield and milk lactose concentration *in vivo*, lending confidence to the use of lactose synthetase as a measure of cellular activity.

The above techniques, although useful in determining mammary development, are either limited to external examination or are invasive. Their application to large animals is generally impractical. Whole organ imaging techniques, for example X-ray computed tomography (X-ray CT) and magnetic resonance imaging (MRI) are now becoming available to the non-clinician. MRI has found widespread usage as a tool for clinical diagnosis in women and limited use with goats (Fowler *et al.*, 1990 and 1991b) and bovine mammary tissue (Sejrsen *et al.*, 1986; Stelwagen *et al.*, 1990) although the bovine studies used excised udders as the size of the imaging equipment was not sufficient for whole animal studies. These techniques provide information on the mammary glands overall size and also quantify parenchymal and stromal tissue. Although potentially useful, these techniques are currently restricted by limited resolution, size of imaging equipment available and cost.

1.2.2 Foetal Development

Unlike most organs, the mammary gland undergoes the majority of its morphogenesis postnatally, however the basic structure is laid down prior to birth.

Stages in the embryonic development of the mammary glands have been studied in many species (Myers, 1916, 1917; Ballinsky; 1950). Although there is a great difference in number and morphology of mammary glands between species, the basic sequence of events during foetal development, follows a similar course. Primordial thickening of the ventrolateral aspect of the epidermis appears very early in

embryonic life and represents the mammary band and streak. Further proliferation of this line gives rise to the mammary line (Turner, 1956), an epidermal thickening extending between fore- and hindlimb buds. Yet further cellular proliferation of the mammary line gives rise to the mammary hillock, and finally the mammary bud as a result of mesenchymal induction (Cunha and Hom, 1996). This sequence of events occurs between day 32 to 43 in the cow, 21 to 28 in the pig and day 35 to 49 in the human (Anderson, 1985). The mammary bud symbolises an important transitional stage in the development of the gland, and is the main structure from which all the mammary glands arise. Cell proliferation then leads to one or more primary sprouts, each primary sprout forms two secondary sprouts which develop a lumen, so that a partial duct system has been established by birth (Cowie *et al.* 1980). Stroma, or connective tissue and an extensive fat pad, forms support for this partially developed duct system and is derived from the mesenchymal layer surrounding the rudimentary gland during foetal development (Mepham, 1987).

1.2.3 Pre-First Conception

From birth to puberty the development of the mammary gland is relatively slow, isometric with the rest of the body, and characterised by the progressive proliferation of the duct system laid down prior to parturition (Astwood *et al.*, 1937). With the onset of ovarian activity, a new phase of more rapid growth occurs, usually some time before first oestrus (Cowie, 1949, Sinha and Tucker 1969). During this phase of positive, allometric growth, the gland increases rapidly in females with intact ovaries (Cowie, 1949). The type of development which takes place at this time appears to be species dependent and, presumably relates to the hormonal milieu. For example, Myers (1916) observed a marked increase in ductal development in rats at this time and more extensive mammary growth, comparable to early pregnancy has been reported for the dog (Cowie *et al.*, 1980).

1.2.4 During Pregnancy

At the time of first conception, the gland consists of little more than a partially developed duct system, surrounded by an extensive fat pad; at the beginning of subsequent pregnancies the gland may be more developed. Mammary growth during gestation (mammogenesis), accounts for the most significant proportion of total growth in most species. For example, it has been estimated, by quantification of mammary DNA, that approximately 59 % of the total mammary growth in the rat (Griffith and Turner, 1961), 78 % in the mouse (Brookreson and Turner, 1959) and 66 % in the rabbit (Lu and Anderson, 1973) occurs during this phase of the reproductive cycle.

Mammary growth, in terms of cell number is slow at the beginning of pregnancy, but accelerates as gestation progresses (Anderson, 1985). This pattern of cell proliferation during gestation has been described as exponential for rats (Munford, 1963), mice (Knight and Peaker, 1982b,d) and also for the hamster, guinea pig, rabbit, goat, sheep and cow (Sheffield and Anderson, 1985). Few data are available for pigs, however, Hacker and Hill (1972) showed little change in total mammary DNA for the first 50 days of gestation followed by a five fold increase between day 50 and parturition in sows. These results are contrary to those of Weatherford (1929), who, on the basis of cytological studies, suggested that the mammary gland did not increase its cellular population during the second half of gestation. However this study was based on cell counts of mammary slices using a light microscope and would not be expected to be as accurate as analysis of total mammary DNA. Interestingly, studies in both rats and mice (Grahame and Bertalanffy, 1972; Knight and Peaker, 1982d) clearly indicated that the proportion of cells undergoing mitosis declined during the second half of gestation. Therefore, in order to maintain an exponential increase in cell number, either the time taken for the cells to divide must decrease, as proposed by Knight and Peaker (1982d), or the rate of cell death must be reduced. Histological studies have shown that this increase in cell mass results in a rapid increase in the size and complexity of the mammary ductal system, with the formation of true lobular-alveolar tissue and depletion of the fat pad (Forsyth, 1982). Towards the end of gestation, as the gland is undergoing final

preparations to secrete milk, individual epithelial cells undergo hypertrophy (Foster, 1977), perhaps as a consequence of milk synthesis.

1.2.5 During Lactation

Proliferation of the mammary secretory cell population during gestation is uniform across mammalian species, however, for a number of species, cellular proliferation continues into lactation. Although Weatherford (1929) concluded that cellular proliferation in the rat was rare after mid-pregnancy, subsequent DNA quantification has shown this to be incorrect. Jeffers (1935) observed occasional mitosis in the rat's mammary gland during lactation and estimated that this proliferation continued throughout the suckling period. This observation was clarified by Greenbaum and Slater (1957), who saw a transient mitotic surge on the second day post-partum in the rat, after which the cell population remained constant for the remainder of lactation. A similar increase in DNA_t has been described for the mouse (Traurig, 1967a), and Knight and Peaker (1982b) described an exponential rate of cellular proliferation between parturition and day 5 of lactation; which resulted in a 100 % increase in DNA_t (Knight and Peaker 1982b). Increases in total mammary DNA during lactation have been reported to account for 41 % of the total in the rat (Griffith and Turner, 1961), 44 % in the rabbit (Lu and Anderson, 1973), 50 % in the guinea pig (Nelson *et al.*, 1962) and 6 % for the hamster (Sinha *et al.*, 1970). Mammary cell proliferation during lactation for ruminant species is less clear; it may account for up to 23 % of total mammary DNA in the goat and 2 % in the sheep (Anderson, 1975a), DNA concentration (mg/g) remained constant, during early lactation in the cow (Baldwin, 1966). However, the study of Baldwin (1966) did not record udder dry mass and so total DNA could not be calculated. DNA concentration is not an accurate measure of cell number as both cell size and mammary adipose tissue would greatly affect it.

The fact that cell proliferation occurs whilst the gland is synthesising milk, raises a fascinating problem: which cells are undergoing mitosis? Milk production is thought to be dependent on a whole functioning alveolus, this contains approximately

50 epithelial cells (Nagato *et al.*, 1980) in addition to myoepithelial cells and stromal tissue. It would appear that functioning alveoli contain fully differentiated cells *i.e.* the cells of a given alveolus differentiate in an all-or-nothing fashion (Keenan *et al.*, 1970) so either all the epithelial cells in any one alveolus are temporarily arrested to undergo mitosis, as suggested by Franke and Keenan (1979), or there is a stem cell population. Devore (1977) identified a population of actively dividing primitive cells in peri-parturient rat mammary tissue and more recently there has been evidence of true stem cells present in all parts of the mammary gland (Medina and Smith, 1990). However, exactly which cells are proliferating is still unclear.

Milk production is a function of not only mammary epithelial cell number, but also the combined activity of these cells. It is widely accepted that cells have to be differentiated before they are capable of synthesising milk. Determining the specific activity of enzymes involved in milk synthesis may be used to estimate the ability of the gland to synthesise milk (see also Section 1.2.1).

After lactational performance has reached a peak, these yields cannot be maintained indefinitely by suckling or by milking artificially. A period of involution appears to be essential to allow the gland to redevelop before the onset of the next lactation (Wheelock and Dodd, 1969). As previously stated, milk yield depends on the number and activity of mammary secretory cells and therefore a declining milk yield can be a result of a progressive loss of cells, a decline in cellular activity or a combination of both. Decline of the secretory cell population has been reported as the prime cause of declining milk yields in goats (Knight and Peaker, 1984b) but a loss of both cells and cellular activity of the remaining cells is cited as the cause in mice (Shipman *et al.*, 1987). The mammary epithelial cells regress to a state that is less differentiated but they remain attached to the extracellular matrix, where extensive remodeling occurs (Politis *et al.*, 1990). The extracellular matrix plays a major role in the maintenance of the functioning epithelial cell population (Streuli *et al.*, 1991; Talhouk *et al.*, 1991). Proteases have been implicated in the disruption of the interactions between the extracellular matrix and epithelial cells which results in programmed cell death, apoptosis (Talhouk *et al.*, 1991). Apoptosis is a controlled, energy dependent process of active self destruction triggered by gene expression

(Hurwitz and Adashi, 1993) and is distinct from necrosis which usually results from toxic agents or ischemia (Wyllie *et al.*, 1980).

1.3 HORMONAL ASPECTS OF LACTATION

Regulatory controls of mammary gland function include specific hormones and growth factors that have been shown to influence, either separately or in concert, the proliferation, differentiation and subsequent involution of the mammary gland. These regulatory signals reach the mammary tissue not only via the traditional endocrine route through the blood supply, but also by autocrine synthesis of specific growth factors by the mammary cells themselves. In addition, there is local interaction between cell types. Such a paracrine pathway involving epithelial-stromal interactions has been demonstrated by a number of investigators (Sakakura, 1991; Woodward *et al.*, 1993).

In the past, blood hormone levels were considered to be proportional to the biological response. However, as information about the nature of the interactions between hormones and their target tissues has accumulated, this view is considered too simplistic. It is now recognised that receptor number and sensitivity as well as hormone interactions have to be considered.

1.3.1 Mammary Differentiation

Mammary gland differentiation, which includes cell proliferation and also activation of genes specific to milk synthesis is under the control of a multitude of hormone and growth factors; these have been extensively reviewed (see Cowie *et al.*, 1980). Briefly, oestrogens and progesterone synergise with the anterior pituitary hormones (growth hormone and prolactin) to evoke mammary development during puberty and pregnancy (Hart and Morant, 1980). The pituitary hormone, prolactin, appears to be particularly important in sheep, since if the prolactin inhibitor bromocriptine is injected, no mammary growth occurs (Hooley *et al.*, 1978). However, mammogenesis in pregnant, hypophysectomised sheep was only fractionally

reduced (Buttle *et al.*, 1979) and in similarly treated rats, no effect was noted (Anderson, 1975b) as long as the placentae were intact. The placental hormone responsible was determined as placental lactogen, a peptide hormone similar to both prolactin and growth hormone.

Increased foetal number and hence placental mass, has been correlated with increased mammary gland weight in sheep (Rattray *et al.*, 1974) and goats (Hayden *et al.*, 1979). In mice, this relationship only appears to be true for up to eight foetal-placental units after which there is no increased effect in mammary development and in rats, maximum mammary development appears to occur with only three (Anderson 1975b). The rat is known to lick its own nipples during pregnancy and it has been proposed that this may stimulate prolactin release, as preventing them from doing this leads to smaller mammary glands (Roth and Rosenblatt, 1968; Knight, 1984).

In order to produce the branching morphology critical to the function of the mammary gland, it is clear that cell division must be appropriately patterned. The autoradiographic work of Bresciani (1971) indicated that oestrogens and progesterone accelerate the rate of cell division in different cell populations of the mammary gland. More recent work suggests that growth factors, such as transforming growth factor-beta (TGF-β), tumour necrosis factor-alpha (TNF-α) and epidermal growth factor (EGF) can regulate end bud and ductal growth at the local level (Coleman and Daniel, 1990; Ip *et al.*, 1992; Rosen *et al.*, 1994). Insulin has been implicated in mammary growth, based on the observations that this hormone is required for the survival of post-natal mammary tissue from many species *in vitro*, and that insulin is a necessary stimulus for DNA synthesis in explants of murine mammary tissue (Topper and Freeman, 1980). However, alloxan, which induces diabetes in rats, had little effect on lobulo-alveolar growth, which would not be expected if insulin was involved in this species (Sud, 1971). In addition, insulin does not stimulate or maintain lobulo-alveolar growth in whole mammary gland explants taken from immature mice (Wood *et al.*, 1975). Rather than insulin itself, structurally related molecules may be involved in mammary growth. Bovine mammary tissue has been shown to synthesise insulin like growth factors (IGFs) (Campbell *et al.*,1991),

and these have been shown to increase bovine mammary cell proliferation *in vitro* acting at the local level (McGrath, *et al.*, 1991).

1.3.2 Lactogenesis and Maintenance of Lactation

Lactogenesis has been defined as the initiation of milk secretion (Cowie *et al.*, 1980). Although a certain degree of confusion has developed due to the definition of the timing of the event, it is generally accepted that the secretion of milk at the time of parturition is called lactogenesis. The eutherian mammary gland is required to be functional at the time of parturition in order to secrete copious quantities of milk. Association of specific hormones with this process is difficult due to the complex interplay of endocrine systems, with marked species differences, however this has been extensively reviewed by Cowie *et al.*, 1980. The minimal hormonal requirement for lactogenesis appears to be a decrease in progesterone along with an increased secretion of prolactin, adrenocorticotropic hormone (ACTH) which stimulates secretion of glucocorticoids, and oestrogens (Tucker, 1985).

Kuhn (1977) argued that the dramatic decrease in serum progesterone noted at the time of parturition is the trigger initiating milk synthesis. It would appear that the decline in progesterone concentration must act in the presence of pituitary hormones, particularly prolactin and adrenal corticoids (Delouis *et al.*, 1980; Forsyth, 1983).

The process of suckling induces a rise in serum prolactin concentration within minutes in the rat, the elevated levels declining on cessation (Grosvenor and Whitworth, 1974). The coupling of prolactin release with milk removal would indicate the importance of prolactin for the maintenace of lactation, at least in the rat. The results of exogenous prolactin treatment during late lactation in rats suckling foster litters prevented the expected decline in milk yield (Flint *et al.*, 1984a); however studies conducted in mid-lactation were more variable (Thatcher and Tucker, 1970). Treatment with exogenous prolactin in ruminant animals has very little effect on milk yield once lactation is established despite a resultant elevation in systemic prolactin (Smith *et al.*, 1974; Jacquemet and Prigge, 1991).

Growth hormone may have a direct action in mammary tissue in cows (Feldman *et al.*, 1993) and rats (Rosen *et al.*, 1994). This is contrary to previous work which reported that growth hormone did not act directly on the bovine mammary gland, (Gertler, *et al.*, 1983) but only via insulin-like growth factor (IGF-I) produced by the liver (Campbell and Baumrucker, 1986). Mammary growth hormone receptors in the cow have been found in cells of the ductal epithelium but not secretory cells (Hauser *et al.*, 1990) which may suggest that milk synthesis is stimulated by growth hormone via a paracrine mechanism (Wilde and Hurley, 1996). There is however, no doubt that the growth-promoting effects of growth hormone are also mediated through the activation of specific target genes, a process that requires a signal transduction pathway leading from the cell surface receptor to the nucleus (Rotwein *et al.*, 1994). As stated above, one of the key targets for growth hormone action is IGF-I which enhances growth in rats (Guhler *et al.*, 1988) and mice (Mathews *et al.*, 1988) by stimulating gene expression (Bichell *et al.*, 1992).

The importance of the thyroid hormones for the maintenance of lactation was established over 50 years ago (see Blaxter *et al.*, 1949). However relatively little is known about its action. Serum concentrations of triiodothyronine (T_3) and thyroxine (T_4) are reduced during lactation in both cattle (Cowie *et al.*, 1980; Tucker, 1985) and rats (Valverde and Aceves, 1989), and as milk yields increase, serum thyroxine concentrations decrease (Vanjonack and Johnson, 1975). Thyroxine is the predominant secretory product of the thyroid gland, and it is this hormone that penetrates the cell before being deiodinised to T_3 which binds to a receptor in the chromatin of the nucleus (Selkurt, 1984). The T_3-receptor complex results in the formation of a specific mRNA and ultimately, a change in the rate of protein synthesis (Selkurt, 1984). However, administration of either exogenous T_3 or T_4 in cattle, stimulates lactational performance, although results are variable (Tucker, 1985). Assuming that it is the mother's intention to provide a quantity and quality of milk to maximise the likelihood of survival of her offspring, this would imply that the reduction in serum thyroxine noted during lactation is a limit to milk synthesis. Van Haasteren *et al.* (1996) proposed that this reduction in thyroxine concentration was a direct result of the high plasma corticosterone levels also noted during lactation

(Walker *et al.*, 1992) suppressing thyrotrophin (TSH) hormone and hence thyroxine release. It has also been suggested that serum thyroxine concentration is lower during lactation as a result of an increased rate of clearance, for instance by loss in the milk (Oberkotter and Rasmussen, 1992). However, the reduced serum thyroxine levels observed by the food restricted rats of Oberkotter and Rasmussen (1992) may be a result of the elevated corticosterone concentrations which would occur (Hart, 1983) in order to enhance endogenous body tissue loss to support lactation (Pine *et al.*, 1994a). As previously stated, raised corticosterone levels have been reported to suppress thyroxine release (Van Haasteren *et al.*, 1996) and this may have occured in addition to the increased thyroxine clearance rate. Clinical trials using hypothyroid women suggested that thyroxine and prolactin were antagonistic at the level of the mammary gland (Edwards *et al.*, 1971), and Cowie *et al.* (1980) pointed out that similar symptoms could equally occur with hyperthyroidism. More recent work would suggest that prolactin is controlled, to at least some extent, by thyrotrophin-releasing hormone (TRH); this hormone appears to positively affect prolactin secretion only over the first few days of lactation in the rat (Hill *et al.*, 1993; Van Haasteren *et al.*, 1996).

The lactating animal's capacity to produce milk is determined by a combination of the growth and development of the mammary gland and also the animal's ability to supply the necessary substrates for milk synthesis. This nutrient partitioning is also largely under the influence of the endocrine system and the literature associated with this control will be discussed below.

1.4 METABOLIC AND NUTRITIONAL ASPECTS OF LACTATION

The functional mammary gland is a highly metabolically active organ and although it confers no special advantage to the animal, it makes tremendous demands on the mother when secreting maximally. This demand is clearly illustrated when the modern dairy cow is examined. Dairy cows often have peak milk yields in excess of 40 kg/day, which would imply a daily secretion rate of approximately 2 kg of lactose and 1.5 kg each of protein and fat. As impressive as this is, the daily milk output is

only approximately 8 % of the dairy cow's body weight; rodents on the other hand have a milk producion as much as 20 % of their total body weight (Grigor *et al.*, 1987; Pine *et al.*, 1994c). However, the relative cost of lactation in terms of protein, expressed as ideal protein requirement of lactation relative to minimal requirements to satisfy obligatory losses occuring within the body was found to be similar for both the cow and rat (Jessop, 1996a). The increased metabolic demands imposed by the mammary gland are met by a number of adaptations in the general metabolism of the whole animal.

The increased nutrient demand is primarily met by an increase in the food intake of the lactating female. This has been confirmed for cattle (Garnsworthy 1988), pigs (Mullan *et al.*, 1989) and rodents (Williamson, 1980; Naismith *et al.*, 1982; Pine *et al.*, 1994a,b,d). In order to maximise food absorption and nutrient utilisation of this increased food intake there is an associated hypertropy of the digestive tract (Hammond and Diamond, 1992; Pine *et al.*, 1994a), liver (Vernon, 1988; Pine *et al.*, 1994a) and heart (Williamsom, 1980) however the kidney mass appears to be unchanged during lactation (Butler-Hogg *et al.*,1985). This increase in organ mass is accompanied by an increase in the absorptive capacity and enzyme activity per unit weight in the digestive tract and liver respectively (Hammond and Diamond, 1992; Hammond and Diamond, 1996; Vernon, 1988) and also an increased cardiac output (Davis and Collier, 1985).

1.4.1 Nutrient Partitioning

The mammary gland is in competition with the other body tissues for this increased supply of nutrients, however survival of the offspring is accorded an extremely high priority and the mother partitions the nutrients not required for her own survival to the mammary gland. This active partitioning of nutrients towards the mammary gland, and away from the peripheral tissues, for example skin, adipose tissue and skeletal muscle during lactation has been termed homeorhesis (Bauman and Currie, 1980). Although these co-ordinated changes in maternal metabolism direct nutrients towards the mammary gland there are times when dietary nutrients fall short

of the mammary gland's requirement. Nutrient shortfall is observed during early lactation in the well-fed dairy cow (Garnsworthy, 1988) and also lactating rats (Pine *et al.*, 1994a,b,d) as milk yield increases at a much faster rate than food intake. Lactating rodents (Naismith *et al.*, 1982; Friggens *et al.*, 1993; Pine *et al.*, 1994a,b,c) and pigs (Mahan and Mangan, 1975) offered a protein-energy imbalanced diet do not show the dramatic increase in food intake normally associated with early lactation. Under such conditions, mobilisation of the mother's protein, fat and mineral reserves help to make up the difference but in severe cases, *e.g.* protein-energy malnutrition, lactational performance is compromised.

In both ruminants and rodents, the onset of lactation is associated with reductions in lipogenesis (Sinnet-Smith *et al.*, 1980; Vernon *et al.*, 1981). This is a result of an increased rate of triglyceride hydrolysis (Zammit, 1988) and an impaired activity of lipoprotein lipase, which is involved in fatty acid uptake from circulating lipoproteins (Vernon *et al.*, 1981). In the mammary gland, however, the activity of this hormone is increased in order to facilitate the uptake of milk fat precursors by the epithelial cells (Mendelson *et al.*, 1977).

The mineral content (calcium, phosphorous, sodium and potassium) of milk is relatively high and therefore high yielding animals, both ruminant and rodent, place a significant demand on the mother to supply these elements. The maternal adaptations to supply these minerals appears to be twofold: improved efficiency of intestinal absorption, and mobilisation from skeletal stores (Block, 1988). It has been estimated that up to 38 % of total bone calcium may be lost during lactation (Halloran *et al.*, 1980) and up to 19 % of the calcium present in rat's milk may be derived from skeletal stores (Brommage, 1989).

Unlike adipose tissue which tends to be stored ready for future mobilisation, available protein reserves are associated with functioning proteins. However, it has been estimated that between 200 to 250 g protein /kg body protein is available for lactation in the rat (Alison and Wannemacher, 1965; Pine *et al.*, 1994a) and the cow (Botts *et al.*, 1979). The primary site of protein mobilisation is reported to be the

skeletal muscle (Swick and Benevenga, 1977; Bryant and Smith, 1982; Millican *et al.*, 1987) and the secondary site, the skin (Cherel *et al.*, 1991).

From the above it is apparent that lactation depends on a number of highly co-ordinated changes in the animal's metabolism so that the mammary gland is able to synthesise the quantity of milk of a sufficient quality to suckle her rapidly growing offspring. Such adaptations in tissue metabolism have to be under controls of adequate sensitivity to react to the changing requirements of the mammary gland. The endocrine system is intimately involved with this regulation of nutrient partitioning. Existing concepts are based almost exclusively on relating metabolical events with serum hormone concentrations. However, as argued above, simple hormone profiles do not adequately describe endocrine regulation at the tissue level as individual tissues may regulate the number and sensitivity of hormone receptors. Endocrine control is further complicated by known synergisms that are not represented by serum hormone concentrations. The major anabolic hormone in both rodents and ruminants is insulin (Vernon, 1988) and therefore it is initially surprising that lactation is associated with hypoinsulinaemia (Williamson, 1980). However, insulin suppresses both lipolysis and proteolysis and stimulates glucose uptake by the mammary gland, peripheral muscle and adipose tissues (Selkurt, 1984); this would put the peripheral tissues in direct competition with the mammary gland for nutrients, which may be detrimental to milk synthesis. Instead there is substantial evidence, for rodents at least, that the mammary gland increases its sensitivity to insulin (Burnol *et al.*, 1987) thereby obtaining a competitive advantage. This advantage is thought to be further enhanced by the peripheral tissues becoming less responsive to insulin in lactating rats (Burnol *et al.*, 1986b) and sheep (Vernon *et al.*, 1985; Vernon *et al.*, 1990). In additon to the mammary gland, the liver also maintains its sensitivity to insulin (Burnol *et al.*, 1986a) in order to promote lipogenesis (Williamson, 1980). The ruminant mammary gland is not sensitive to insulin (Vernon *et al.* 1983), however the hypoinsulinaemia observed during lactation in ruminants is still beneficial as it promotes an increased rate of hepatic gluconeogenesis.

Glucagon antagonises many of the actions of insulin and would therefore be expected to aid the partitioning of nutrients for utilisation by the mammary gland.

The serum insulin to glucagon ratio is thought to be important in the regulation of ruminant liver metabolism (Basset, 1975), increasing gluconeogenesis and ketogenesis. Serum glucagon concentration does not vary during lactation in cattle (Sartin *et al.*, 1985), however the reduced insulin concentration will result in a decreased insulin : glucagon ratio which would be expected to have an effect on bovine liver metabolism. Although the rodent's mammary gland is not sensitive to glucagon (Robson *et al.*, 1984) adipocyte sensitivity has been reported to increase *in vitro* (Zammit, 1988).

The serum growth hormone concentration rises rapidly during lactation in ruminants (Bauman and Elliot, 1983; Collier *et al.*, 1984, 1993; Vernon, 1989), but not rodents (Schalch and Reichlin, 1966). Growth hormone in lactating cattle has been shown to promote gluconeogenesis *in vitro* (Pocius and Herbein, 1986) and also partition milk precursors towards the mammary gland *in vivo* (Peel *et al.*, 1981). Growth hormone has been shown to have an intrinsic lipolytic affect (Bines and Hart, 1978), and to directly inhibit insulin stimulated lipogenesis in lactating cows (Marinchenko *et al.*, 1992). Thus its role in mobilising body fat as a source of energy and diverting dietary nutrients away from tissue synthesis may be considerable. As previously discussed, the galactopoietic effects of growth hormone are thought to act primarily through IGF-I in ruminants (Campbell and Baumrucker, 1986); however only extremely high concentrations of IGF-I increased the rate of lipogenesis in adipose tissue. As a result Vernon (1989) suggested that this insulin-like growth factor was acting via insulin and not IGF-I receptors and this hormone would therefore be expected to have very little effect on nutrient partitioning.

Given the elevated levels of prolactin observed during lactation in rats (Grosvenor and Whitworth, 1974) and ruminants (Jacquemet and Prigge, 1991) this hormone may be expected to play a role in nutrient partitioning. However, although prolactin stimulates amino acid uptake and metabolism of the mammary epithelial cells in rats (Vina *et al.*, 1981) and acts indirectly on the adipose tissue of rodents (Vernon and Flint, 1983), its actions as a homeorhetic regulator remain uncertain.

The fundamental feature of white adipose tissue is its ability to store lipid. It is innervated by the sympathetic system which stimulates the release of catecholamines from both the adipose tissue itself and also the adrenals (Trayhurn and Ashwell, 1987). Catecholamines actively promote lipid mobilisation through the activation of lipase (Trayhurn and Ashwell, 1987). Brown adipose tissue, the primary function of which is to produce heat, either for thermoregulation or in relation to the regulation of energy balance, is innervated by the sympathetic system to a greater extent than white adipose tissue (Trayhurn and Ashwell, 1987). In contrast to white adipose tissue, the sympathetic system suppresses thermogenesis in brown adipose tissue, sparing substrates for milk synthesis in addition to reducing the effects of the metabolic heat production associated with lactation (Trayhurn, 1985).

The concept that non-essential dietary nutrients are partitoned towards the mammary gland at the expense of other tissues is well established. It is also clear that the mammary gland not only receives nutrients of dietary origin, but these may also be supplemented by mobilisation of endogenous fat, protein and minerals. The implementation of the necessary mechanisms required to meet the high nutrient demands of the mammary gland is primarily under the control of the endocrine system and is also well understood, although the exact pathways of hormone action are less clear.

1.4.2 Nutrition and Lactation

From the above, it is clear that the animal accords a high priority to lactation and undergoes a number of metabolic adaptations to partition both dietary and endogenous nutrients towards the mammary gland at the expense of other tissues. Lactation would, therefore, be expected to be sensitive to both the quantity and quality of food offered. During lactation, food intake, which is controlled by the hypothalamus, increases (unless it is restricted by environmental circumstance or artificially) thereby increasing the nutrient pool. In dairy cows this increase is progressive over the first few weeks of lactation, with peak intake occuring approximately 8-12 weeks post-partum (Bauman and Elliot, 1993), and rats increase

their food intake over the first 8-10 days of lactation (Pine *et al.*, 1994a,b,c). Studies in humans have reached conflicting conclusions as to whether maternal dietary intake does (Edozien *et al.*, 1976; Forsum and Lonnerdal, 1980) or does not (Prentice *et al.*, 1980) affect lactational performance. However, the obvious experimental limitations incurred with clinical trials draws into question many of the results, as the studies are often incomplete and the nutrient demand for lactating humans is very low anyway. Animal trials, on the other hand, are relatively straightforward and, as a consequence there is a plethora of literature concerning feeding and milk production (see Broster and Thomas 1981; Garnsworthy, 1988; Friggens, 1991) of which only some will be discussed here. Not many of these studies have investigated the effects of intake during early lactation, a critical period when the level of nutrient intake is often below that of output and a time when mammary cell mass may still be increasing. Surprisingly, few experiments have examined the sensitivity of the mammary secretory cell population to dietary effects, given the importance of the mammary gland for milk production.

Broster *et al.*, (1969) studied the effects of two fixed levels of feeding using dairy heifers over the first 18 weeks of lactation. The animals offered the lower food intake did not show the increase in milk yield seen in the animals offered the high food intake regime over the first five weeks of lactation. The decline in milk yield began in week five, three weeks earlier, than for those animals offered the high intake regime. Interestingly, one group of animals was offered the high plane of nutrition after nine weeks of the low intake regime, and milk yields increased, though not to the levels seen in animals offered this level of intake from parturition. These data clearly indicate that the bovine mammary gland is sensitive to the level of dietary intake. But it is not certain from this work, whether it was the protein or energy intake that had the primary effect. Neither was it clear whether the reduced milk yield was a result of a lack of substrate available for milk synthesis or a suppression of mammary development during this period. A compensatory rise of milk yield on changing from low to higher protein levels was also noted in dairy heifers during early lactation (Oldham *et al.*, 1979). Once again, differences in digestible energy intake between groups offered these two diets confounds the energy and protein response, making it

impossible to isolate the protein effects. When offered a low protein diet during early lactation, ewes also showed a dramatic suppresion of milk yield, which was rapidly reversed by re-alimentation with a high protein concentration diet (Robinson *et al.*, 1979), in agreement with the work of Oldham *et al.* (1979).

The daily milk yield of rodents is, not surprisingly, very difficult to determine accurately, although a variety of techniques have been discussed by Linzell (1972). As a result many studies assess milk production from daily weight gains of litters of a standard size. Although this a good indication of the dam's lactational ability, a number of points should be remembered: firstly, litter weight gain is a product of both milk quality which is influenced by diet (Crnic and Chase, 1978; Grimble, 1981; Pine *et al.*, 1994c) as well as milk volume. Secondly, milk production has been reported to be a function of suckling intensity (Flint *et al.*, 1984) and, therefore comparisons of similar work which have used different litter sizes should be made with caution. Finally, litters from dams offered poor dietary regimes are likely to be smaller than those offered good ones. As a result, their metabolic costs for maintenance will be different and so comparisons between groups of the same age may not be accurate.

Restricting voluntary food intake to 40 % of *ad libitum* during lactation has been reported to suppress litter weight gain in rats significantly (Grigor *et al.*, 1987). These authors also reported a reduction in specific activity of mammary enzymes involved with milk synthesis, indicating a reduction in secretory cell activity. The restriction on dietary intake was only imposed after lactation was well established (day 7) when the mammary gland secretory cell population would be expected to be stable (Griffith and Turner, 1961) and therefore it is possible that the reduction in milk yield is entirely due to the reduced cellular activity. Since the cell mass was not measured this is not certain.

In response to a dilution in dietary protein content, non-lactating rats (Musten *et al.*, 1974) and growing pigs (Kyriazakis *et al.*, 1990) increase their food intake, presumably to maintain total protein intake, whereas paradoxically, lactating rats decrease their intake (Naismith *et al.*, 1982; Grigor *et al.*, 1987; Friggens *et al.*, 1993; Pine *et al.*, 1994abc). In the studies of Naismith *et al.*, (1982) and Grigor *et al.*

(1987), the reduction in dietary protein was replaced with carbohydrate, and it has been argued that this increase in carbohydrate may have had an influence on food intake (Friggens *et al.*, 1993). The work of Pine *et al.* (1994a,b,c) replaced the dietary protein with both carbohydrate and fat so the ratio of these two constituents remained 2.3:1 in all dietary regimes, which were also isoenergetic and intakes were still depressed, indicating that it was the low protein to energy ratio that was responsible. As food intake would be expected to increase during early lactation, the suppression of food intake observed when a low protein concentration diet is offered would be expected to lead to a reduction in lactational ability. This has been shown for rats (Sampson *et al.*, 1986; Grigor *et al.*, 1987; Friggens *et al.*, 1993; Pine *et al.*, 1994a,b,c) and humans (Sampson and Jansen, 1984a). Lactating rats, re-alimentated with a high protein concentration diet after 6 days of protein restriction have been shown to rapidly increase their food intake and lactational performance to levels similar to control rats offered a high protein concentration diet from day 1 of lactation (Pine *et al.*, 1992). However, as the diets in all the above studies with a low protein to energy ratio suppressed intake, it has not been possible to ascribe the reduced lactational performance to either protein or energy intake.

The mammary gland synthesises milk, and the studies discussed above clearly indicate that this gland is sensitive to nutrient intake. In addition, low protein concentration diets have been reported to have a negative effect on milk protein concentration (Crnic and Chase, 1978; Grimble, 1981; Pine *et al.*, 1994c) which would be detrimental to the growth of their offspring. But none of the above studies adequately investigated the dietary effect on the mammary gland itself, in terms of secretory cell number and function.

Rats offered a high protein concentration diet, pair fed at 30 % of *ad libitum* intake from week 5 of age showed no difference in mammary DNA concentration (mg/g dry mass) at mid-lactation, 16 weeks of age (Park *et al.*, 1994); however mammary dry mass was not reported and so it is not possible to determine the effect of nutrient restriction on total mammary DNA from this study. One study that attempts to assess the effects of nutrition during lactation on mammary development during lactation has been reported by Knight and Peaker (1982d). The effects of a 24

hour fast immediately post-partum on murine mammary gland composition and lactational performance were investigated. Both total DNA, and hence secretory cell number, and RNA were significantly reduced by day 2 of lactation when compared with non-fasted mice. But, by day 7 of lactation, there were no significant differences between these 2 groups for either total DNA or RNA, indicating that the mammary glands of the fasted rats had undergone compensatory growth. Interestingly, the daily growth rates of the standardised litters (9 pups) from these fasted dams were reduced for a much longer period of time, indicating that although mammary mass had increased, lactational performance had not. Offering a low protein concentration diet during lactation has been shown to decrease total mammary DNA by 19 % and RNA by a similar proportion (Pyska and Styczynski, 1979b). Unfortunately the data for food intake were not reported in this study and it is not possible to determine whether this was a protein or energy effect.

1.5 THESIS OBJECTIVES

From the above discussion it is apparent that our understanding of mammary gland development, from foetus through to lactation, is well understood in terms of physical development and endocrine control. The nutrient requirements of the lactation cycle have been well described, and to a lesser degree, the role of endogenous reserves in supplementing the diet are known. However, our understanding of how the mammary gland itself responds, in terms of the primary components of lactational ability, namely, secretory cell number and activity, to protein and energy intake during lactation are unknown. As a result, it was decided to address this gap in our understanding, by undertaking a number of rat trials. The aim of this thesis was to assess the sensitivity of the mammary gland, in terms of secretory cell number and activity to both protein and energy intake during lactation.

Four trials were done and are described in chronological order in the ensuing chapters, the individual objectives of each are summarised below. Rats were chosen as the experimental animal for a number of reasons:

- they are easy to handle, allowing relatively large numbers to be housed during each experiment, and cross-fostering of pups allows a high, but consistent lactational demand to be imposed;
- the ease of removing the entire mammary gland and carcass dissection;
- the short timescale of their reproductive cycle allows rapid accumulation of data;
- they are relatively cheap, therefore analysis of groups of animals reduces the effect of individual variation;
- the rat has a substantially elevated nutrient requirement during lactation, which allows a substantial range of food restriction regimes to be employed.

Trial 1 tested the hypothesis that protein undernutrition and re-alimentation during lactation would have no effect on secretory cell number or activity. This was clearly demonstrated to be incorrect but the suppression of food intake, and hence energy intake associated with offering a low protein to energy ratio diet prevented a definitive answer as to whether this was an energy or protein effect.

Trial 2 assessed the possibility of using the anti-histamine receptor antagonist, cyproheptadine, to increase the voluntary food intake of rats offered the low protein to energy ratio diet. Although, the drug did significantly increase the voluntary food intake of these rats, the effect was not long term, and a further method had to be devised to split the effects of dietary energy and protein on mammary development during lactation.

The primary objective of trial 3 was to separate the effects of dietary protein and energy intake on mammary secretory cell number and activity, after an initial period of protein restriction by rationing protein and energy allowances seperately.

This experiment also investigated the effects of protein and energy intake on mobilisation of maternal body reserves during lactation.

Trial 4 investigated the hypothesis that the increase in mammary cell mass observed on realimentation with a diet of high protein concentration, after a period of protein restriction was a result of mammary cell proliferation and not through a reduced rate of cell death.

CHAPTER 2

TRIAL 1

Mammary Sensitivity to Protein Restriction and Re-alimentation

The basis of this Chapter has been published.

British Journal of Nutrition (1996) **76.** 423-434. (Appendix III)

2.1 ABSTRACT

This study tested the influence of protein undernutrition and realimentation on mammary gland size and secretory cell activity in lactating rats. During gestation female Sprague-Dawley rats were offered a high protein concentration diet (H; 215 g crude protein (N×6.25; CP)/kg dry matter (DM)); litters were standardised to 12 pups at parturition. During lactation, two diets were offered *ad libitum*, one of high protein concentration, diet H, and one low (L; 90 g CP/kg DM). Lactational dietary treatments were *ad libitum* supply, of either diet H (HHH) or diet L (LLL) for the first 12 days of lactation, or diet L transferring to diet H on either day 6 (LHH) or 9 (LLH) of lactation. On day 1, 6, 9 and 12 of lactation rats from each group ($n \geq 6$) were used to estimate mammary dry mass, fat, protein, DNA and RNA. The activities of lactose synthetase enzyme (*EC* 2.4.1.22) and Na^+,K^+-ATPase (*EC* 3.1.6.1.37) were also measured.

Rats offered a diet considered protein sufficient (H) from day 1 of lactation showed a decrease in mammary dry mass and fat but an increase in DNA, RNA and protein on day 6, after which there was no further change except for mammary protein which continued to increase. However, rats offered diet L, showed a steady loss in mammary mass and fat throughout the 12 day lactation period and no change in mammary DNA, RNA or protein. Rats previously protein restricted for either the first 6 or 9 days of lactation, had their mammary dry mass and mammary fat loss halted and showed a rapid increase in mammary DNA, RNA and protein upon re-alimentation. Lactose production in group HHH as measured by lactose synthetase activity was similar on day 1 and 6 of lactation, after which a significant increase was seen. Protein restricted rats showed no change in lactose synthetase activity during the 12 day experimental period. Changing from diet L to H led to a significant increase in lactose synthetase activity to levels comparable with those offered diet H from day 1.

These results show that rats offered a protein restricted diet during lactation suffer mammary underdevelopment, but this may be rapidly reversed by realimentation with a diet of high protein concentration.

2.2 INTRODUCTION

Milk yield is a function of the number and activity of secretory cells present in the mammary gland. Development of this gland has been shown to occur during both gestation and early lactation, although development during lactation varies between species. For example, it has been shown that increases in total DNA during lactation accounts for 41% of the total DNA in the rat (Griffith and Turner, 1961), 24% in the mouse (Brookreson and Turner, 1959), 44% in the rabbit (Lu and Anderson, 1973) and 6% for the hamster (Sinha *et al.*, 1970). Data for ruminant species is less clear. However mammary cell proliferation during lactation may account for up to 23% of the total in the goat (Knight and Peaker, 1984a), 2% in the sheep (Anderson, 1975a). Equivalent data for cattle, despite their economic importance, is unreported.

Altering a lactating animal's diet can change both the yield and composition of milk produced (Broster *et al.*, 1969; Knight and Peaker, 1982d; Pine *et al.*, 1994a,c). Despite the plethora of literature on mammogenesis, both during gestation and lactation, little attention has been given to the effects of nutrition and in particular protein malnutrition on mammary development. Undernutrition in virgin rats (Srivastava and Turner, 1966) and during gestation (Sykes *et al.*, 1948; Rosso *et al.* 1981) can reduce mammary weight and therefore lactational performance, as can offering protein deficient diets during gestation (Pyska and Styczynski, 1979a) and lactation (Pyska and Styczynski, 1979b). The primary objective of this experiment was to assess the sensitivity of the rat mammary gland, in terms of mammary cell number and cellular activity, to protein undernutrition and re-alimentation during lactation.

2.3 MATERIALS AND METHODS

2.3.1 Experimental protocol

Seventy-seven multiparous (second parity) female Sprague-Dawley rats (B & K Universal Ltd., Hull) weighing on average (300 (SEM 2.45) g) were housed in a room regulated at 22 °C with relative humidity between 45-65% and with a light period from 07:00-19:00 hours for a minimum of two weeks before breeding. During

this period they were offered a standard rat chow (B & K Universal Ltd., Hull) *ad libitum*. At the appropriate time, females were placed individually in a wire bottomed cage with a proven male breeder. Day 1 of gestation was the morning on which mating was confirmed through the presence of a vaginal plug, after which the females were caged individually in solid-bottomed, plastic cages for the remainder of the experiment.

From day 1 of gestation the females were offered *ad libitum* a high protein diet (H; 215 g crude protein (N×6.25; CP) / kg dry matter (DM)), formulated to meet NRC (1978) requirements for vitamins and minerals, until parturition which was designated day 1 of lactation (Table 2.1). On this morning, litters were standardised to 12 pups to ensure a uniform and high lactational demand. Litters that could not be standardised to 12 by cross fostering with pups born on the same day were removed from the trial, along with their mothers.

Table 2.1 Composition of diets H and L (g/kg DM)

	High (H)	**Low (L)**
Casein - methionine (99:1 w/w)	215	90
Maize Oil	192	230
Corn Starch - Sucrose (2:1 w/w)	444	530
Vitamin Mix[†]	50	50
Mineral Mix[†]	50	50
Maize Flour	40	40
Choline chloride	7	7
Antioxidant[‡]	0.013	0.013
Emulsifier[§]	2	2

[†] Mineral and vitamin mixtures were formulated to meet N.R.C. (1978)requirements.
[‡] Antioxidant - butylated hydroxy toluene (g/kg fresh weight).
[§] Emulsifier - egg lecithin (g/kg fresh weight).
All diets were isoenergetic with constant carbohydrate energy to fat energy ratio (2.3:1)

Diet Analysis:	Protein (g CP/kg DM)	H 216 (SEM 0.12);	L 89 (SEM 0.08)
	Gross energy (MJ/kg DM)	H 21.3 (SEM 0.07);	L 21.2 (SEM 0.09)

During lactation two diets were offered *ad libitum*, both formulated to meet NRC (1978) requirements for vitamins and minerals, one of high protein concentration, diet H, and one of low (L; 90 g CP /kg DM) protein concentration. The protein source for both diets was casein supplemented with DL-methionine (99:1, w/w). All diets were formulated to provide 21 MJ gross energy (GE) kg^{-1} DM with a constant carbohydrate energy : fat energy ratio of 2.3:1. Lactational dietary treatments were *ad libitum* supply of either diet H (HHH; *n=6*) or diet L (LLL; *n=8*) for the first 12 days of lactation or diet L with transfer to diet H after either 6 (LHH; *n=6*) or 9 (LLH; *n=7*) days lactation. This dietary allocation produced four groups of females (LLL, LLH, LHH and HHH) the first letter representing the dietary treatment from day 1 of lactation to day 6, the second from day 6 to 9 and the third from day 9 to 12.

Dam body weights and feed intakes were recorded at the same time each day throughout the experiment as were standardised litter weights. All females were given free access to fresh drinking water.

Dams were killed by decapitation on either day 1(*n=6*), 6 (L; *n=6*, H; *n=7*), 9 (LL; *n=7*, LH; *n=6*) or 12 of lactation when the mammary gland was dissected from all animals.

2.3.2 Lactose synthetase (E.C. 2.4.1.22) activity

Rates of lactose synthetase activity were determined *in vitro,* based on the method described by Vonderhaar (1977) using fresh mammary tissue. Mammary tissue (approx. 400mg) from the right inguinal gland was removed immediately following decapitation, chopped (20 μm slices) and homogenized using a hand-held, all glass homogeniser in 2 ml of 0.02 mol/l Tris-HCl buffer, pH 7.4 at 4 °C, containing 0.01 mol/l $MgCl_2$ and 0.001 mol/l β-mercaptoethanol.

Palmiter, (1969c) reported a loss in lactose synthetase specific activity for murine mammary tissue after several hours. As it was not possible to slaughter animals and assay their homogenates at precisely the same time each day, it was necessary to correct for this possible loss in activity. In addition, samples from the

first 5 rats slaughtered were frozen at -15 °C, for 7 days prior to assay, while the assay technique was improved. In order to correct for the possible loss of activity in both the fresh samples and the 5 frozen samples, an enzyme stability study was developed. Freshly dissected tissue was homogenised, an aliquot was removed and assayed at time zero and hourly for 7 hours, the homogenate being kept at 4 °C between sampling points. The remainder was immediately frozen at -15 °C and assayed daily for 12 days.

2.3.3 Measurement of mammary oxygen consumption

Oxygen consumption rates of mammary tissue were measured polarographically as previously described by Pine *et al.* (1994b). The difference between the initial O_2 consumption (total respiration) and that following oubain treatment (Na^+,K^+-ATPase independent respiration) was termed the Na^+,K^+-ATPase-dependent respiration. The percentage inhibition of the original O_2 consumption associated with Na^+,K^+-ATPase activity was calculated using the ratio of Na^+,K^+-ATPase dependent respiration to total respiration.

2.3.4 Mammary Analysis

Mammary dry mass was determined by freeze drying to a constant mass. The freeze dried mammary gland was stored at -20 °C for approximately 5 weeks before being cooled in liquid nitrogen and ground to a fine powder using a pestle and mortar, and analysed for total protein, DNA, RNA and fat. Protein was determined by the method of Lowry *et al.* (1951) using bovine serum albumin as a standard. DNA was quantified using PicoGreen Nucleic Acid Quantitation Reagent (Molecular Probes, Europe BV, The Netherlands), a fluorescent stain for quantifying double stranded DNA. RNA was extracted using a total RNA Isolation Reagent (Advanced Biotechnologies Ltd., Leatherhead, U.K.) and quantified by measuring absorbance at 260 nm.

Mammary fat was extracted using petroleum spirit (40 °C to 60 °C) using a Soxhlet flask and thimble.

Cell number was calculated from total mammary DNA (DNA_t) using the equation of (Winick and Noble,1965):

$$\text{Cell Number} = \frac{DNA_t \text{ (mg)}}{6.2 \times 10^{-9}}$$

2.3.5 Statistical Analysis

The results were analysed by two way analysis of variance, with dam day 1 gestation body-weight as a covariate (Genstat5), and calculation of least significant differences; *t* tests were used to compare sample means between dietary treatment groups.

2.4 RESULTS

2.4.1 Maternal body-weight changes, feed intakes and litter-weight gains

The results for the lactation groups LLL, LLH, LHH and HHH are summarised in Table 2.2, Figure 2.1 and Figure 2.2.

By day 6 both groups, LLL and HHH had lost body weight, but feeding the low-protein diet resulted in a continued weight loss and by day 12 these females were significantly ($P<0.01$) lighter than those continuously fed Diet H which had effectively regained their body weight. Dams previously offered Diet L for either the first 6 or 9 days of lactation, showed a rapid and significant ($P<0.05$) increase in body weight when transferred to Diet H returning to a weight comparable with group HHH by day 12.

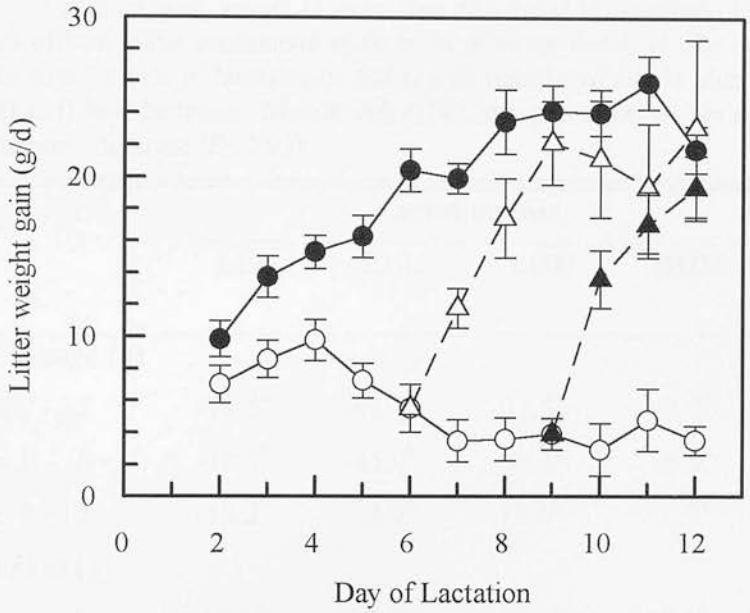

Figure 2.1 Daily litter-weight gain (g) of rats offered either a low (L)- or high (H)-protein diet for the first 12 days of lactation, or diet L with transfer to diet H after either 6 or 9 days lactation: (–○–), LLL (*n8*); (–▲-), LLH (*n7*); (–△-), LHH (*n6*); (–●–), HHH (*n6*). Values represent a mean and SEM, using dam day 1 gestation weight as a covariate.

Food dry matter intakes of Diet L and H followed a similar course for the first 3 days of lactation, after which they diverged. Consequently, this resulted in a much greater total feed intake (g DM) for females offered Diet H compared to those offered Diet L ($P<0.01$; Table 2.2). Groups LHH and LLH showed an immediate increase in food intake when offered Diet H after days 6 and 9 respectively, and by day 12, the 12 day accumulated intake for females from group LHH was only slightly lower and not significantly different from those in group HHH.

Table 2.2. Maternal body weight change, feed intake and standardised litter weight gain of rats offered either continuous application of either diet L (LLL) or diet H (HHH) for the first 12 days of lactation or diet L with transfer to diet H after either 6 (LHH) or 9 (LLH) days lactation. Means with different superscripts within the same row are significantly different ($P<0.05$).

	Lactation diet				
	LLL	**LLH**	**LHH**	**HHH**	**Pooled SED**
Dam weight change (g)					
Days 1 - 12	-98.5^{b}	-42.1^{a}	-21.6^{a}	-5.2^{a}	19.04
Days 1 - 6	-40.3^{b}	-45.0^{b}	-35.1^{b}	-6.9^{a}	8.82
Days 7 - 12	-58.2^{b}	2.9^{a}	13.6^{a}	1.7^{a}	16.90
Dam feed intakes (g)					
Days 1 - 12	161.0^{b}	193.0^{bc}	272.0^{ac}	310.0^{a}	36.6
Days 1 - 6	69.5	69.6	69.5	101.5	86.1
Days 7 - 12	75.0^{b}	124.0^{bc}	185.0^{ac}	209.0^{a}	29.2
Litter weight gains (g)					
Days 1 - 12	59.7^{b}	94.3^{b}	166.4^{a}	215.3^{a}	20.10
Days 1 - 6	38.8^{b}	35.2^{b}	52.1^{b}	75.6^{a}	7.11
Days 7 - 12	20.9^{b}	59.1^{b}	114.2^{a}	139.7^{a}	17.24

Lactational performance was estimated by the weight gain of a standardised litter and closely reflected dry matter intake (Figure 2.1 and 2.2). The greater supply of both dietary energy and protein received by dams offered Diet H allowed their litters to achieve a greater weight gain during lactation when compared to those offered Diet L. Changing from Diet L to H after days 6 and 9 allowed both groups LHH and LLH to increase lactational performance as measured by daily litter weight gain, with significant improvements in litter gain being seen within 24 hours of the dietary change.

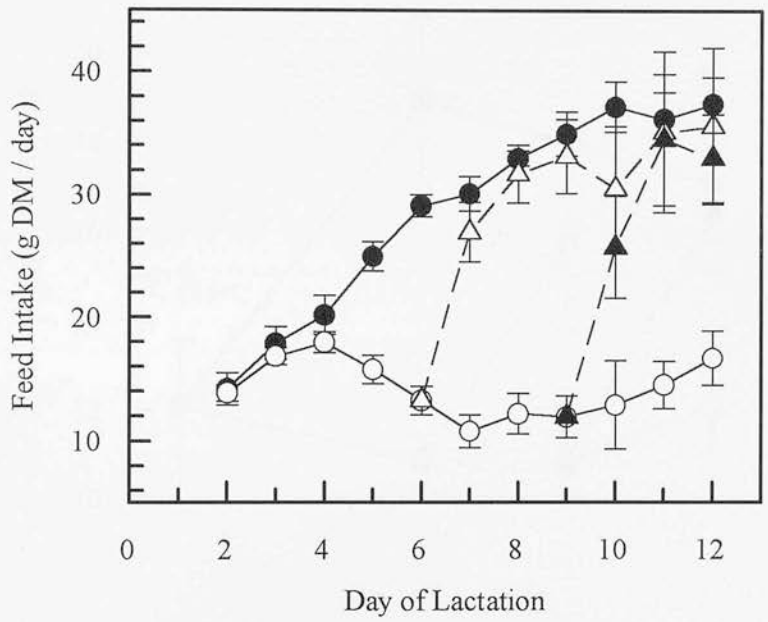

Figure 2.2 Daily food dry matter intake (g) of rats offered either a low (L)- or high (H)-protein diet for the first 12 days of lactation, or diet L with transfer to diet H after either 6 or 9 days lactation: (-○-), LLL (*n8*); (-▲-), LLH (*n7*); (-△-), LHH (*n6*); (-●-), HHH (*n6*). Values represent a mean and SEM, using dam day 1 gestation weight as a covariate.

2.4.2 *Effect of lactational dietary treatments on mammary gland composition during lactation*

The main effects of the lactation dietary treatments on mammary gland dry mass and composition are shown in Table 2.3 and Figure 2.3.

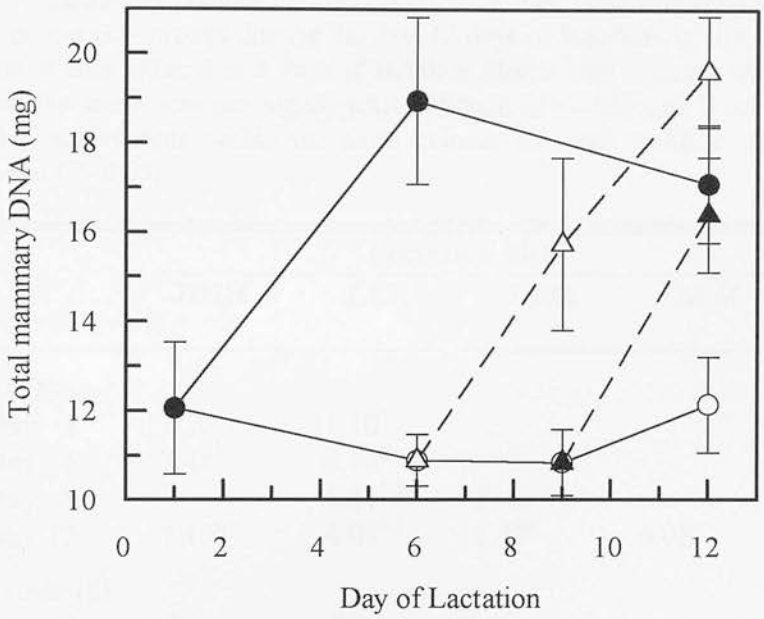

Figure 2.3 Total mammary gland DNA (mg) of rats offered either a low (L)- or high (H)- protein diet for the first 12 days of lactation, or diet L with transfer to diet H after either 6 or 9 days lactation: (–○–), LLL; (–▲-), LLH; (–△-), LHH; (–●–), HHH. Values represent a mean and SEM, using dam day 1 gestation weight as a covariate.

Mammary dry masses and total mammary fat values significantly decreased in all groups until day 6 of lactation, after which losses continued in rats on diet L but were halted in those rats offered diet H and those transferred from diet L to H after both 6 and 9 days of lactation.

Table 2.3 Mammary composition on days 1, 6, 9 and 12 of rats offered either a high (H)- or low (L)- protein diet for the first 12 days of lactation, or diet L with transfer to diet H after either 6 or 9 days of lactation.Means with different letter superscripts within the same row are significantly different ($P<0.05$) and those with different symbol superscripts within the same column for each variable are significantly different ($P<0.05$).

	Lactation diet				
	HHH	**LLL**	**LHH**	**LLH**	**Pooled SED**
Dry mass (g)					
Day 1	11.10^{*}	11.10^{*}			
Day 6	$7.44^{\dagger}$	$6.44^{\dagger}$			0.88
Day 9		$5.31^{\dagger,\ddagger}$	7.05		
Day 12	$7.15^{a,\dagger}$	$4.03^{b,\ddagger}$	6.47^{a}	6.05^{a}	
Total protein (g)					
Day 1	1.94^{*}	1.94			
Day 6	2.564^{*}	1.902			0.28
Day 9		1.776^{a}	2.624^{b}		
Day 12	$3.262^{a,\dagger}$	1.674^{b}	2.899^{a}	2.575^{a}	
Total Fat (g)					
Day 1	8.89^{*}	8.89^{*}			
Day 6	$4.70^{\dagger}$	$4.21^{\dagger}$			0.74
Day 9		$3.17^{\dagger,\ddagger}$	4.21		
Day 12	$3.68^{\dagger}$	$2.03^{\ddagger}$	3.08	3.27	
Total RNA (mg)					
Day 1	55.8^{*}	55.8			
Day 6	$104.7^{\dagger}$	62.7			16.52
Day 9		53.6^{a}	97.5^{b}		
Day 12	$119.1^{a,c,\dagger}$	43.4^{b}	132.9^{a}	86.9^{c}	
RNA : DNA					
Day 1	5.13	5.13			
Day 6	5.79	6.07			1.16
Day 9		5.25	6.67		
Day 12	7.01^{a}	3.68^{b}	6.74^{a}	5.09^{a}	

Dams offered diet H showed a rapid and significant increase in DNA, RNA and protein between day 1 and 6 of lactation, after which the mammary protein continued to increase but there was no further significant change in total DNA or RNA content. However, rats offered diet L showed no change in mammary protein

or nucleic acids during the 12 day lactation period, but when these rats were offered diet H after both days 6 and 9 days, groups LHH and LLH respectively, they showed a significant increase in nucleic acid and protein content.

2.4.3 Mammary cell activity

The responses of the mammary enzyme, lactose synthetase, to lactational dietary treatment and the oxygen consumption and associated Na^+,K^+-ATPase activity of the mammary gland are given in Table 2.4.

The activity of lactose synthetase decreased in rats offered diet L and by day 9 activities were significantly ($P<0.05$) lower than those on day 1. However, on day 12 the activities had increased to levels not different from those on day 1. Rats offered diet H showed no significant change in lactose synthetase activities over the first 6 days of lactation, after which activities significantly increased ($P<0.05$). Changing from diet L to H on either day 6 or 9 led to a significant increase ($P<0.05$) in lactose synthetase activity to levels comparable with those fed diet H from day 1.

The enzyme stability study showed no significant loss of specific activity over 7 hours at 4 °C. However, the activities of the five samples kept for 7 days at -20 °C showed a decline with time and were corrected using linear regression analysis (loss in specific activity = 78.8 d.p.m day^{-1}; $r^2 = 0.93$; (equivalent to 9.8% day^{-1} of initial activity)).

The mammary gland total respiration, Na^+,K^+-ATPase-dependent and independent activity of the mammary gland tended to decline in rats fed both diets H and L over the 12 day lactation period, although the reductions were not statistically significant at the 5% level; Table 2.4. Rats previously offered diet L showed a significant ($P<0.05$) increase in total respiration rate from 690 to 1199 nmol O_2 mg^{-1} DNA min^{-1} when offered diet H after 6 days. However, it was only a temporary increase and by day 12 the total cellular oxygen consumption was similar to those rats offered diet L for the full 12 days. The increase in total respiration rate from 549 on

day 9 to 841 nmol O_2 mg^{-1} DNA min^{-1} seen on day 12 when diet H was offered after 9 days of *ad libitum* diet L was not significant.

Table 2.4 Mammary lactose synthetase activity (nmoles lactose formed mg^{-1}DNA min^{-1}) and oxygen consumption (nmol mg^{-1} DNA min^{-1}) on days 1, 6, 9 and 12 of rats offered either a low (L)- or high (H)- protein diet for the first 12 days of lactation, or diet L with transfer to diet H after either 6 or 9 days of lactation. Means with different letter superscripts within the same row are significantly different ($P<0.05$) and those with different symbol superscripts within the same column for each variable are significantly different ($P<0.05$).

	Lactation diet				
	LLL	**LHH**	**LLH**	**HHH**	**Pooled SED**
Lactose synthetase activity					
Day 1	80.8^{*}			80.8^{*}	
Day 6	$54.6^{*,\dagger}$			114.3^{*}	21.96
Day 9	$35.1^{a,\dagger}$	169.2^{b}			
Day 12	$74.9^{b,*,\dagger}$	194.0^{a}	188.0^{a}	$226.7^{a,\dagger}$	
Total respiration					
Day 1	714			714	
Day 6	690			673	167.3
Day 9	549^{a}	1199^{b}			
Day 12	644	841	854	577	
Na^{+},K^{+}-ATPase-dependent respiration					
Day 1	155			155	
Day 6	132			122	56.7
Day 9	110^{a}	390^{b}			
Day 12	$173^{a,c}$	$193^{a,c}$	$261^{b,c}$	100^{a}	
Na^{+},K^{+}-ATPase-independent respiration					
Day 1	560			560	
Day 6	558			551	124.8
Day 9	439^{a}	809^{b}			
Day 12	471	647	593	477	
Percent inhibition					
Day 1	22.0			22.0	
Day 6	17.3			16.3	4.51
Day 9	19.9^{a}	33.4^{b}			
Day 12	$24.9^{a,b}$	$23.4^{a,b}$	30.6^{b}	16.7^{a}	

Oxygen consumption attributable to Na^+,K^+-ATPase activity (Na^+,K^+-ATPase dependent) followed a similar pattern to the total cellular oxygen consumption, but the increases seen on refeeding were more pronounced. As a consequence, the increases in Na^+,K^+-ATPase independent respiration were smaller on refeeding diet H and no significant difference was seen between treatments or time.

2.5 DISCUSSION

The primary objective of this study was to assess the sensitivity of the rat mammary gland to dietary protein restriction and realimentation during lactation, in terms of mammary cell number and cellular activity. The results show that the mammary gland is extremely sensitive to realimentation, and that rapid increases in both cell mass and cellular activity were seen. The rapidity of the response was remarkable and has not previously been reported. Similarly, the responsiveness of the gland to nutrition at a relatively advanced stage of lactation is an important observation.

Voluntary intake of a diet with a low protein to energy ratio is considerably reduced in rats when compared to that of one with a higher protein to energy ratio. This results in an impaired lactational performance, as indicated by the daily litter weight gain of standardised litters in agreement with previous experiments from our laboratory (Pine *et al.* 1994*a*,b,*d*). A reduced intake of a diet with a low protein concentration (diet L) must lead to a protein and hence also an amino acid deficiency, in addition to a shortfall of other nutrients and energy, when compared with the performances of rats receiving diet H.

DNA is located almost entirely within the nucleus and its mass is constant within the diploid nucleus of the rat mammary cell (Griffith and Turner, 1957; Tucker and Reese, 1962). Mirsky and Ris (1949) suggested that total tissue DNA content (DNA_t) may be used as an accurate indicator of cell number and therefore development. This was later confirmed by Tucker, (1987). For assessment of

mammary gland secretory mass, the assumption that changes in DNA mass are due only to changes in the mammary epithelial cell population must also be made. Paape and Sinha (1971) showed that the adipose and connective tissue cell population is largely determined before first conception, therefore, any change in DNA_t noted during lactation may be ascribed to the secretory cell population. It is essential to base growth studies on total DNA because changes in cell size greatly affect DNA concentration.

Although feeding diet H resulted in a decrease in mammary dry mass (36%) over the 12 day period, it did promote an increase (58%) in DNA_t and therefore secretory tissue mass. The overall loss of dry mass could largely be ascribed to the loss of fat over this period. Cell number had reached a maximum by day 6, which is in agreement with other data for rats (Griffith and Turner, 1961) and mice (Knight and Peaker, 1982b). Knight and Peaker (1982b,d) suggested an exponential increase in murine mammary secretory cells, from day 12 of gestation until day 5 of lactation with a doubling time of 6 days. As day 6 was the first sample point after parturition it is not possible to determine either the exact time cell number reached a maximum or a doubling time. Feeding diet L, which was considered inadequate in terms of protein but not energy concentration, to dams in group LLL from day 1 of lactation inhibited any increase in the secretory cell population of the mammary gland. Although diet L was isoenergetic with diet H over the 12 day lactation period, the accumulated food dry matter (DM) intake was 48% less than those rats fed diet H for the equivalent period. Thus L rats received approximately 78% less dietary protein but also 48% less gross energy than H rats, and consequently it is not known whether secretory cell mitosis was inhibited due to a protein or energy shortage. Whether this absolute nutrient shortfall acts directly on the gland or indirectly via a disturbance of the dam's metabolic and endocrine processes cannot be determined from this study. However, the accumulated food DM intakes until day 4 of lactation (a period of rapid cell proliferation in the dams of group HHH) were not significantly different for groups HHH and LLL (47.90 (SEM 6.13) g DM and 42.93 (SEM 4.77) g DM respectively) whereas the crude protein intakes were significantly different (15.27 (SEM 1.54)g and

5.10 (SEM 0.40) g; $P<0.001$ respectively) which may suggest protein was the limiting factor.

Carrick and Kuhn (1978) estimated lactose synthetase activities *in vivo* and reported that, like many metabolic parameters, the enzyme exhibits diurnal fluctuations, with synthesis highest during the early morning and lowest during early evening. For this reason, all samples were taken at a similar time during the morning, kept at 4 ^{0}C and analysed as quickly as possible. The daily increase in litter growth rate throughout the 12 day lactation period reflects the demand for milk imposed upon the dam during this period. As the total secretory cell population remained static from day 6, this increase in milk production must be achieved by an increase in milk synthesis per cell. When diet H was offered, the capacity of the cells to synthesise lactose apparently remained constant for the first 6 days of lactation, however it must be remembered that no intermediate sample was taken. Although lactose production per cell remained constant over this period for these rats, cell number was increasing rapidly, and therefore total lactose production per gland was also increasing. The second half of lactation showed a significant, positive increase in lactose synthetase activity and, by day 12, the dams were producing almost 2 g lactose per day. This compares favourably with the data of Carrick and Kuhn (1978) who calculated that Wistar rats fed a standard chow would produce approximately 1.4 g lactose per day, but at peak synthesis rates, when the measurements in the present study were taken, they would be producing 1.6 g per day. The slightly increased production rate, reported in this trial may have been due to a diet containing a higher protein concentration. Mansaray and Grimble (1983) fed Wistar rats a comparable protein concentration diet (200 g/kg DM) to diet H used in this study and, from their measured lactose synthesis rates on day 14 of lactation, it can be calculated that their rats would have been producing 1.92 g lactose per day. Lactational malnutrition has been reported to suppress lactose synthesis (Carrick and Kuhn, 1978; Wilde and Kuhn, 1979; Mansaray and Grimble 1983) and these results confirm this. Lactose synthetase specific activity was significantly ($P<0.05$) suppressed by day 9 when compared with day 1, though this significance was lost by day 12.

Since mammary RNA is intimately related to the biosynthesis of protein, this nucleic acid may be used as an indication of the cells synthetic potential (Winick and Noble, 1965). Dams offered diet H for the entire 12 day period showed a significant increase in total mammary RNA (RNA_t) over the first 6 days after which no further increase was seen, in agreement with Pine *et al.* (1994d). The pattern of development was similar to that of DNA and, as a result, the RNA content per cell, expressed as the ratio of RNA:DNA did not change over the lactational period. Dams offered diet L, throughout the experimental period showed a slight, but not significant, reduction in RNA_t (22%), so by day 12 they had approximately 64% less RNA than dams from group HHH on the same day, implying a dramatic shortfall in protein synthetic capacity. Of perhaps more interest is the RNA to DNA ratio which was not significantly reduced on days 6 or 9, when compared to day 1. This may indicate that the cells were able to preserve an adequate quantity of RNA for protein synthesis, but the actual rate of protein synthesis was limited by substrate availability. By day 12 the cellular RNA had significantly decreased in the LLL group suggesting that the mammary epithelial cells now had a reduced protein synthetic capacity. Although the total mammary protein values for this group were significantly less than those for group HHH ($P<0.01$) on day 12, there was no significant difference within group LLL over the 12 day period (Table 2.3). This may suggest that by day 12 of lactation, the secretory cells reduced capacity for protein synthesis in group LLL had not, as yet, become noticeable. It is however, also possible that milk protein synthesis may have been impaired as previous authors have demonstrated that dietary protein restriction during lactation reduces milk protein concentration (Crnic and Chase 1978; Sturnman *et al.* 1986; Pine *et al.* 1994c) and cellular protein synthesis in these cells had been spared. Specific activity of the enzyme lactose synthetase was not dramatically suppressed on day 12 when compared with day 9. In fact it showed a slight increase in activity, reflecting the increase in food intake, though neither of these increases were significant. This also suggests that the cells were restricted through lack of substrate rather than RNA.

Mammalian cellular mechanisms require oxygen. Two of the most demanding are protein synthesis and the membrane transport system Na^+,K^+-ATPase, which plays

an essential role in the transport of both sugars and amino acids across cell membranes (Milligan and Summers, 1986). The mammary epithelial cells of lactating rats are highly active cells and it would be expected that differences in cellular activity would be reflected in the utilization of greater quantities of oxygen and the proportion of tissue energy expenditure associated with Na^+,K^+-ATPase activity. The above results do not support this and are in agreement with those of Pine *et al.* (1994b), (Table 2.4). Mammary epithelial cells of dams offered only diet H or only diet L, had similar tissue respiration rates and the proportion associated with Na^+,K^+-ATPase activity remained unchanged. It should, however, be remembered that group HHH showed considerable hypertrophy in terms of cell number which would result in a considerable increase in total and Na^+,K^+-ATPase-dependent energy expenditure. Dams offered diet L with transfer to diet H after 6 days (LHH) and 9 days (LLH) showed a temporary increase in both total respiration and the oxygen consumption attributable to Na^+,K^+-ATPase, suggesting a period of rapid cellular activity, perhaps in advance of an increase in cell number.

Dams initially protein restricted, by offering diet L during lactation and then reoffered diet H on day 6 or 9 (groups LHH and LLH respectively) showed a rapid, significant increase in food intake to levels comparable with those dams offered diet H from day 1. This rapid increase in nutrient supply in turn led to an equally rapid and significant increase in daily weight gain of standardised litters from these groups. This improvement in lactational performance was due to a rapid, positive response in mammary cell mass, lactose synthetase activity, RNA_t, and a temporary increase in total cellular respiration rate and Na^+,K^+-ATPase-dependent energy expenditure. This would suggest that the mammary gland of rats, whose endogenous labile protein reserves may be assumed to have been depleted (Pine *et al.*, 1994d), is still capable of becoming fully functional after nine days of severe dietary protein restriction during lactation. These results also show that although mammary cell proliferation is complete by day 6 of lactation in dams offered a diet of favourable protein to energy ratio, it is still possible to trigger mitosis in dams offered a diet with a low protein to energy ratio after this time. Realimentation with a diet of high protein concentration, diet H, led to an increased food intake, which would lead to a greater supply of

metabolites for utilization by the mammary gland. This may also be further enhanced by an increased blood flow to the gland (Williamson, 1984). It is not clear what is inhibiting mitosis and reducing secretory cell activity in the dams offered a high energy, low protein diet. This low protein to energy ratio diet resulted in a significantly reduced dry matter intake which led to a period of severe protein, and hence amino acid restriction. This would have certainly led to an amino acid deficiency, however it is not known whether this amino acid deficiency or any other nutrient / energy shortfall inhibited mitosis and suppressed cellular activity. Therefore, it is unclear whether the reversal was due to the greater supply of amino acids, energy yielding or other nutrients acting directly on the gland; or through other factors *e.g.* the production of a blood borne signal, hormone or metabolite. One peptide inhibitory factor, mammary derived growth inhibitor (MDGI), has been shown to inhibit mitosis in several mammary cell lines (Bohmer *et al.*, 1984) and is expressed *in vivo* in differentiated cells (Kurtz *et al.*, 1990). This has led Burgoyne and Wilde (1994) to the suggestion that MDGI may be a regulator of cell proliferation. This work, however, did not assay for MDGI or any other factor.

In summary, it can be concluded that lactating rats offered a diet with a low protein to energy ratio reduced food DM intake, and thus subjected themselves to a period of severe protein restriction, which resulted in poor lactational performance. Poor lactational performance was a consequence of an absence of mammary secretory cell proliferation during lactation combined with a low activity of these cells. When a diet with a higher protein concentration was offered, food intake increased rapidly and there was a corresponding increase in lactational ability. This increase in lactational ability was a result of rapid secretory cell proliferation and a marked increase in cellular activity.

CHAPTER 3

TRIAL 2

Protein-Energy Malnutrition: Responses to the Histamine Receptor Antagonist Cyproheptadine During Growth or Lactation

The basis of this Chapter has been submitted to the British Journal of Nutrition for publication (Apendix III).

3.1 ABSTRACT.

The study examines the effects of the histamine receptor antagonist, cyproheptadine, on the voluntary food intake and corresponding weight change of both lactating and weanling rats over a 12 day period.

Twenty-five multiparous rats (310 (SEM 9.1) g) were offered *ad lib.* one of two isoenergetic diets, one of high (H; 215 g crude protein (CP)/kg dry matter (DM)) protein concentration and one low (L; 90 g CP/kg DM) protein concentration. During gestation, rats were offered diet H; litters were standardised to twelve pups at parturition. Lactational dietary treatments were either diet L or diet H for the first 12 days of lactation. In addition, thirty-six weanling rats (145 (SEM 1.0) g) were offered *ad lib.* one of three isoenergetic diets (V; 40 g CP/kg DM; L or H) for a 12 day experimental period. All animals were weighed daily and received a daily injection intraperitoneally (ip) of either saline (S; 150 mol/l) or cyproheptadine (D; 2.5 mg/kg body weight) dissolved in saline for the 12 day period.

Control lactating rats offered diet L (MLS) ate less ($P<0.05$), this resulted in a greater body weight loss ($P<0.05$) and significantly reduced lactational performance when compared with control rats offered diet H (MHS). Food intake, weight change and lactational performance of lactating rats offered diet L and injected with cyproheptadine (MLD) was comparable with rats offered diet H and significantly different from the control group MLS for the first 5 day period but not over the 11 day period. No effect of cyproheptadine was observed in lactating rats offered diet H. Weanling rats offered diets L and H showed no significant differences in cumulative food intake between day 1-6 or day 1-12, however rats offered diet V had significantly reduced intakes when compared with the other groups. Cyproheptadine administered to weanling rats failed to significantly increase food intake when compared with control rats offered the same diet over either 5 or 11 days.

The results suggest that food intake suppression shown by lactating rats offered a low protein concentration diet may be a result of increased brain histamine over the short term. It is suggested that diet V was not sufficiently protein limiting to initiate sufficient body protein mobilisation to significantly increase plasma and hence

brain histidine concentrations in the weanling rats.

3.2 INTRODUCTION.

Voluntary food intake is known to be influenced by many factors. It is well established that food intake can be limited when dietary protein concentration is reduced in the diet of growing and lactating rats (Peng *et al.*, 1972; Peters and Harper, 1985; Pine *et al* 1994a,b,c) and this was confirmed in Trial 1, Chapter 2. It has been suggested that this reduction in voluntary food intake is a result of increased brain histamine concentration acting on central histamine receptors located on the hypothalamus and thereby reducing voluntary food intake (Orthen-Gambill, 1987; Mercer *et al.*, 1989 and Mercer *et al.*, 1994). Rats offered a low protein concentration diet during lactation will supplement the dietary protein deficiency by mobilising labile body protein reserves until deplete, approximately day 8 of lactation (Pine *et al.*, 1994*b,c*). Both skeletal muscle (Pine *et al.*, 1994d) and haemoglobin (Nasset and Gatewood, 1954) have been reported as significant sources of endogenous protein catabolised during periods of dietary protein restriction. Catabolism of both muscle carnosine, a dipeptide of β-alanine and histidine, and haemoglobin, which contains approximately 8% histidine, would be expected to increase plasma histidine concentrations. Indeed, muscle carnosine and haemoglobin levels have been shown to be reduced and plasma histidine concentration increased in rats tube-fed diets of low histidine content (Clemens *et al.*, 1984). Histidine is transported into the brain principally on the large neutral amino acid carrier along with several other amino acids (Pardridge, 1983). A rise in plasma histidine coupled with a reduction of the other indispensable amino acids seen when low protein concentration diets are offered would raise brain histidine levels at the expense of others, including the neurotransmitter precursors tryptophan and tyrosine (Gustafson *et al.*, 1986). Increased brain histidine concentration leads to an increased rate of histamine synthesis, because normal physiological concentrations of histidine do not saturate the enzyme histidine decarboxylase (*EC* 4.1.1.22) in the brain ($K_m=4\times10^{-4}$ M) (Pardridge,

1983; Hegstrand and Simon, 1985). Therefore, it may be assumed that histamine is synthesised in the brain at a rate proportional to intracellular histidine concentration, until saturation is achieved. Histamine receptors are located on the hypothalamus, the brain region intimately involved with food intake regulation (Richelson, 1992). In addition, low protein concentration diets suppress the specific activity of the hepatic enzyme histidine ammonia-lyase ((*EC* 4.3.1.3) also known as histidase) compounding the peripheral histidine concentration increase (Kang-Lee and Harper, 1977; Clemens *et al.* 1984). If brain histamine does suppress food intake, treatment with histamine antagonists would be expected to reverse the suppression and indeed this has been shown to be true for the growing rat (Kaluchy, 1980; Orthen-Gambill, 1987; Orthen-Gambill and Salomon, 1989; Mercer *et al.*, 1994). Unfortunately, these studies have only been conducted over the short term.

The primary objective of this experiment was to assess whether the suppression of voluntary food intake observed during early lactation in rats offered a low protein concentration diet could be reversed by the administration of the antihistamine antagonist, cyproheptadine over a 12 day period.

3.3 MATERIALS AND METHODS.

3.3.1 Experimental Protocol.

Twenty-five multiparous (second parity) and thirty-six female weanling Sprague-Dawley rats weighing on average (310 (SEM 9.1) g) and (145 (SEM 1.0) g) respectively (B & K Universal Ltd., Hull) were housed in a room regulated at 22°C with relative humidity between 45-65% and with a light period from 06:00-18:00 hours. Breeding females were offered a standard rat chow (B&K Universal Ltd., Hull) *ad libitum* for a minimum of two weeks before breeding and the weanling rats offered the same diet for one week. During this period all animals were weighed daily to familiarise them to their routine so as to minimise stresses resulting from handling during the experimental period. At the appropriate time, breeding females were placed individually in a wire bottomed cage with a proven male breeder. Day 1 of

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gestation was confirmed through the presence of a vaginal plug, after which these females were caged individually in solid-bottomed, plastic cages for the remainder of the experiment.

From day 1 of gestation the pregnant dams were offered *ad lib.* a high protein diet (H; 215 g crude protein (N×6.25; CP) kg^{-1} dry matter (DM) see Table 3.1) until parturition which was designated day 1 of lactation. On that morning litters were standardised to 12 pups to ensure a high but uniform lactational demand. Litters which could not be standardised to 12 pups by cross fostering with pups born on the same day were removed from the trial, along with their mothers.

Table 3.1 Calculated and analysed composition of the high- (H), low- (L) and very low- (V) protein concentration diets (g/kg dry matter (DM)).

Diet	H	L	V
Ingredients			
Casein/Methionine[1]	215	90	40
Maize oil	192	229	244
Starch-sucrose	442	529	564
Vitamin Mix	50	50	50
Mineral Mix	50	50	50
Maize starch	43	43	43
Choline chloride	7	7	7
Lecithin	2	2	2
Butylated hydroxytoluene	0.01	0.01	0.01
Analysis			
Protein (g CP/kg DM)	216 (SEM 0.1)	89 (SEM 0.2)	40 (SEM 0.1)
Gross energy (MJ/kg DM)	21.3 (SEM 0.2)	21.6 (SEM 0.1)	21.4 (SEM 0.1)

[1] Casein / *DL*-Methionine (99:1 w/w).

Lactational dietary treatments were either *ad lib.* supply of a high protein concentration diet, diet H, or a low (L; 90 g CP kg^{-1} DM) protein concentration diet, for the first 12 days of lactation. After one week acclimatization, the weanling rats were offered *ad lib.* one of three protein concentration diets, one high; diet H, one low; diet L and the third very low (V; 40 g CP kg^{-1} DM) for a period of 12 days. All

diets were formulated to provide 21.5 MJ gross energy (GE) kg^{-1} DM with a carbohydrate energy to fat energy ratio of 2.3:1 and to meet NRC (1978) requirements for minerals and vitamins. The protein source was casein supplemented with DL-methionine (99:1,w/w). Detailed dietary compositions are given in Table 3.1. The lactating and weanling rat studies were carried out simultaneously.

Lactating rats were injected intraperitoneally (ip) with either cyproheptadine (D; 2.5 mg kg^{-1} body weight (BW) dissolved in 150 mol l^{-1} sterile saline) or physiological sterile saline (S; 150 mol l^{-1}). Drug concentration was varied such that the total volume injected was approximately 0.5 ml. Weanling rats were injected ip with exactly the same dosage rates of either cyproheptadine (D) or physiological saline (S), however the total volume injected was only 0.3 ml. Injections were administered daily, just prior to the dark period.

This experimental regimen led to ten groups: MHS (*n=5*), MHD (*n=5*), MLS (*n=5*) and MLD (*n=4*), WHS (*n=6*), WHD (*n=6*), WLS (*n=6*), WLD (*n=6*), WVS (*n=6*) and WVD (*n=6*) where the first letter refers to the animals age (M; lactating and W; weanling) the second letter refers to dietary treatment and the third to solution injected.

The body weights and feed intakes of both lactating and weanling rats were recorded daily at the beginning of the light period, as were standardised litter weights. All animals were given free access to fresh drinking water.

Animals were killed by decapitation on day 12 when the liver was dissected from all animals.

3.3.2 Hepatic histidine ammonia-lyase (EC 4.3.1.3) specific activity.

Histidine ammonia lyase (histidase) specific activity was determined *in vitro* by measuring the rate of urocanic acid production by a modification of the procedure of Tabor and Mehler (1955). Hepatic tissue (approx. 2 g) was removed immediately following decapitation and thoroughly homogenised using a Potter-Elvehjem, glass / teflon homogeniser in 6ml ice cold buffer (pH 7.4) containing 0.01 mol l^{-1} Tris HCl,

0.014 mol l^{-1} $MgCl_2$ and 0.6 mol l^{-1} KCl. The homogenate was spun for 2 min at 12500×g, the supernatant removed and stored at -80 °C until assayed, approximately 2 weeks later. Thawed samples were centrifuged at 122000×g for 90 min at 4 °C (MSE Prepspin 65 Ultracentrifuge) after which the supernatant was removed and incubated in a shaking water bath for 30 min at 55 °C before cooling on ice. The cooled supernatant was further spun at 66000×g for 30 minutes at 4 °C (MSE Prepspin 65 Ultracentrifuge) to remove thermally denatured proteins. The reaction was initiated by the addition of 30 µl of the final supernatant to 1.5 ml of freshly prepared reaction mixture (pH 9.0) containing 0.007 mol l^{-1} reduced glutathione, 0.01 mol l^{-1} sodium pyrophosphate and 0.05 mol l^{-1} L-histidine in quartz glass cuvettes.

Urocanic acid formation was monitored continuously at 277 nm over a 30 min period at 37 °C using water-jacketed cuvette holders (Beckman DU-65). The molar extinction coefficient of urocanic acid was taken to be 0.0188 mol l^{-1} cm^{-1} (Tabor and Mehler, 1955). Results were expressed as mmol l^{-1} urocanic acid produced per min per mg homogenate protein. Protein was assayed by the method of Lowry *et al.* (1951) using bovine serum albumin as a standard.

3.3.3 Liver Analysis.

Liver dry mass was determined by freeze drying to a constant mass. The freeze dried liver was stored at -20 °C for approximately 3 weeks before being cooled in liquid nitrogen and ground to fine powder using a pestle and mortar, and analysed for total protein. Protein was determined by the method of Lowry *et al.* (1951) using bovine serum albumin as a standard.

3.3.4 *Statistical Analysis.*

Results were analysed by two way analysis of variance, with either dam day 1 gestation body-weight or day 1 treatment body-weight for the weanling rats as a covariate (Genstat5 Release 3.1), and calculation of least significant differences; *t*-tests were used to compare sample means between treatment groups.

3.4 RESULTS.

3.4.1 Maternal food intakes, body-weight changes and litter weight gains of lactating rats.

The results for the lactation groups MHS, MHD, MLS and MLD are summarised in Figures. 3.1, 3.2 and 3.3.

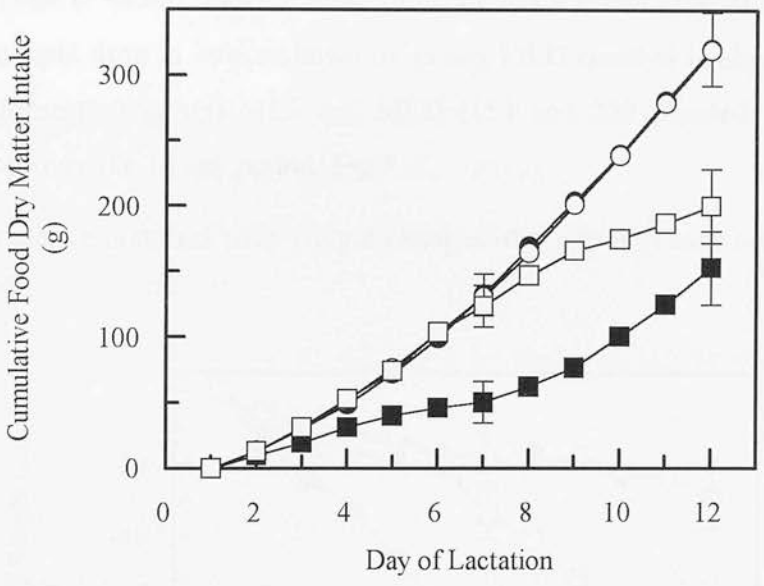

Figure 3.1 Cumulative food dry matter intake (g) of lactating dams (M)- offered either a high (H)- or low (L)- protein diet receiving daily injections of either physiological saline (S) or cyproheptadine (D): (-●-), MHS (*n*5); (-○-), MHD (*n*5); (-■-), MLS (*n*5); (-□-), MLD (*n*4). Values represent a mean with pooled SED, using day 1 gestation body weight as a covariate. Refer to text for detailed account of dietary and drug treatments.

Lactating rats offered diet H ate significantly more, over the 12 day period than those offered diet L, and the histamine receptor antagonist, cyproheptadine had no significant effect on food intake in these rats (MHS and MHD), Figure 3.1. Rats offered diet L and administered with physiological saline (MLS) showed a significantly suppressed intake ($P<0.05$) over the 12 day period when compared with those rats offered diet H, the cumulative intakes being significantly less than groups MHS and MHD by day 6 of lactation. However, the food DM intakes of rats offered

diet L and injected with cyproheptadine (MLD) followed a similar course to those rats offered diet H until approximately day 7 of lactation when intakes began to decrease. The cumulative intake was not significantly less than that of rats offered diet H until day 11 of lactation. As a result of the increased food DM intake shown by group MLD, the mean cumulative intakes of these rats were significantly greater than the means of the control group MLS between day 6 and 11 of lactation. By day 12 of lactation, the mean voluntary food intake of these rats was only 12.51 (SEM 4.0) g significantly less ($P<0.05$) than the mean of group MLS which was 30.56 (SEM 7.4) g. This dramatic drop in intake shown by group MLD resulted in similar total DM intakes of these two groups MLS and MLD (153 and 200 (Pooled SED 28.2) g respectively), over the 12 day period, Fig 3.1.

Cumulative maternal body weight changes of the four groups are summarised in Figure 3.2.

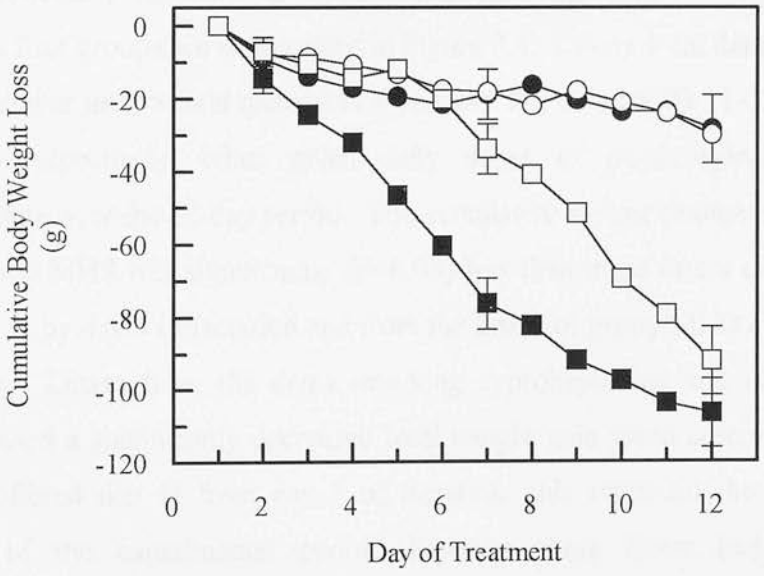

Figure 3.2 Cumulative weight change (g) of lactating dams (M)- offered either a high (H)- or low (L)- protein diet receiving daily injections of either physiological saline (S) or cyproheptadine (D): (-●-), MHS (*n*5); (-○-), MHD (*n*5); (-■-), MLS (*n*5); (-□-), MLD (*n*4). Values represent a mean with pooled SED, using day 1 gestation body weight as a covariate. Refer to text for detailed account of dietary and drug treatments.

The drug cyproheptadine had no significant effect on the maternal body weight change of rats offered diet H. Both groups offered this diet, MHS and MHD lost 28.4 and 30.0 (Pooled SED 8.91) g respectively over the 12 day period. Control rats offered diet L which received daily injections of physiological saline, group MLS rapidly lost body weight and by day 4 they had lost significantly ($P<0.05$) more body weight than the other 3 groups, by day 12 they had lost 28% of their day 1 body weight. However, rats offered diet L and administered with cyproheptadine, MLD, showed a reduced rate of body weight loss until day 7 of lactation, comparable to those rats offered diet H. After day 7 they began to show a dramatic weight loss which continued until the end of the trial. By day 12 they had lost 92.1 (Pooled SED 9.45) g, a weight loss comparable to those rats offered diet L and injected with saline which had lost 106.5 (Pooled SED 9.45) g and a significantly ($P<0.01$) greater weight loss than those rats offered diet H.

Lactational performances, as measured by the growth rate of standardised litters of the four groups are summarised in Figure 3.3. Litters from dams offered diet H showed similar total weight gains, 241.4 and 243.9 (Pooled SED 11.01) g for MHS and MHD respectively, when given daily doses of physiological saline and cyproheptadine over the 12 day period. The cumulative weight change of litters from dams in group MHS was significantly ($P<0.05$) less than those litters of both groups offered diet H by day 3 of lactation and from the litters of group MLD dams by day 6 of lactation. Litters from the dams receiving cyproheptadine and offered diet L (MLD) showed a significantly decreased total weight gain when compared to litters from rats offered diet H from day 3 of lactation, this remained the case for the remainder of the experimental period, however these litters had acquired a significantly greater weight than litters in group MLS by day 6 of lactation, and although daily weight gain in these rats began to decrease after day 9 of lactation their total weight remained significantly greater.

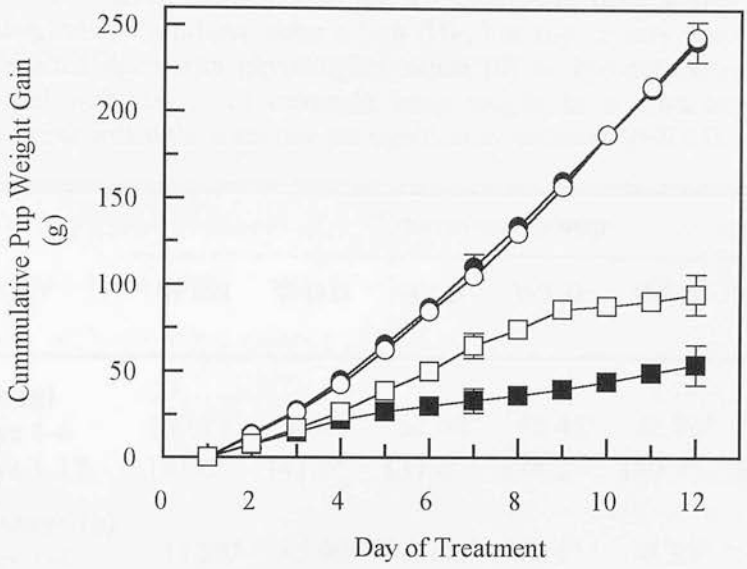

Figure 3.3 Cumulative growth (g) of standardised litters (12 pups) from lactating dams (M)- offered either a high (H)- or low (L)- protein diet receiving daily injections of either physiological saline (S) or cyproheptadine (D): (-●-), MHS (*n*5); (-○-), MHD (*n*5); (-■-), MLS (*n*5); (-□-), MLD (*n*4). Values represent a mean with pooled SED, using day 1 gestation body weight as a covariate. Refer to text for detailed account of dietary and drug treatments.

3.4.2 Food intakes and body-weight changes of weanling rats.

The DM intake and body weight changes of weanling rats over the 12 day experimental period are summarised in Table 3.2. Animals offered diets H or L *ad lib.* and treated with either saline or cyproheptadine ate similar amounts over the 12 day period. Weanling rats offered the very low protein diet, (V) had a significantly ($P<0.05$) reduced voluntary food intake when compared to rats offered diet H or L over the first 6 day period. However, rats offered V and were treated with saline, (WVS) increased their voluntary food intake between day 6 and 12 so that by day 12 total DM intake was similar to that for H or L rats, whereas group WVD showed a significantly ($P<0.05$) reduced 12 day cumulated intake when compared to all other groups.

Table 3.2 Dry matter (DM) intakes (g) and cumulative body weight changes (g) of weanling rats (W)- offered either a high (H)-, low (L)- or very low (V)- protein diet and injected daily with physiological saline (S) or cyproheptadine (D). Means, corrected with day 1 of treatment body weight as a covariate with different superscripts within the same row are significantly different ($P<0.05$).

	Treatment Group						
	WHS	**WHD**	**WLS**	**WLD**	**WVS**	**WVD**	**Pooled SED**
DM Intake (g)							
Days 1-6	60.18^{a}	58.41^{a}	62.68^{a}	55.45^{a}	42.36^{c}	38.70^{c}	2.85
Days 1-12	141.0^{a}	141.0^{a}	137.4^{a}	134.2^{a}	130.4^{a}	100.8^{b}	8.30
Weight Change (g)							
Days 1-6	17.25^{a}	15.99^{a}	13.03^{a}	7.15^{b}	-7.35^{c}	-9.38^{c}	1.96
Days 1-12	39.96^{a}	42.31^{a}	30.76^{b}	27.35^{b}	-5.20^{c}	-3.57^{c}	3.01

Animals from groups offered either diet H or L gained body weight over the first 6 days, however animals from group WLD had a significantly ($P<0.05$) reduced cumulative weight gain over this time period when compared to the other three groups. After 12 days this drug effect had been lost, however groups WHS and WHD had gained significantly more weight than groups WLS and WLD (Table 3.3). Rats offered diet V lost significant body weight over the first 6 day period ($P<0.001$) when compared to that of rats offered diet H or L. Although this weight loss was halted during the second six day period, the initial weight loss was not reversed and the total cumulative weight change was negative and significantly ($P<0.001$) lower that the other groups.

3.4.3 Effect of dietary treatment and drug administration on liver composition.

The effects of dietary treatment and histamine receptor antagonist on liver dry mass and composition for both lactating and weanling rats are given in Table 3.3. Neither diet nor drug treatment affected liver dry mass in the lactating animals; however offering diet L led to a significantly reduced total liver protein mass (Table 3.3). Hepatic histidine ammonia-lyase specific activity was significantly ($P<0.05$) reduced in

the lactating rats receiving saline and offered diet L, when compared to the other three groups. Dietary protein concentration, but not drug administration significantly ($P<0.05$) affected both liver dry mass and total liver protein mass. Animals offered diet H had both the highest dry and protein masses; those offered diet V the lowest masses and those animals offered diet L intermediate masses (Table 3.3). Hepatic histidine ammonia-lyase specific activity was not significantly altered by either diet or drug treatment in the weanling animals (Table 3.3).

Table 3.3 Liver dry mass, total protein (g) and Hepatic histidine ammonia-lyase (*EC* 4.3.1.3) activity (mmol urocanic acid min^{-1} mg^{-1} protein) of both lactating and weanling rats. Lactating rats were offered *ad lib.* diets H and L and weanling rats were offered *ad lib.* diets H, L and V, within each dietary group half received daily injections of physiological saline (S) and half cyproheptadine (D). Means from lactating rats were corrected with day 1 gestation body weigh as a covariate and means from the weanling animals day 1 of treatment body weights. Means with different superscripts within the same row are significantly different ($P<0.05$).

	Treatment group						
	HS	**HD**	**LS**	**LD**	**VS**	**VD**	**Pooled SED**
Lactating (M)[1]							
Dry Mass (g)	5.41^{a}	4.83^{a}	3.25^{a}	3.68^{a}			0.82
Total Protein (g)	3.23^{a}	2.70^{a}	1.65^{b}	1.79^{b}			0.26
Histidase Activity[2]	3.06^{a}	2.97^{a}	0.87^{b}	1.66^{a}			0.72
Weanling (W)[1]							
Dry Mass (g)	2.89^{a}	3.00^{a}	2.25^{b}	2.33^{b}	1.85^{c}	1.89^{c}	0.11
Total Protein (g)	1.62^{a}	1.77^{a}	1.40^{b}	1.46^{b}	1.02^{c}	1.06^{c}	0.06
Histdase Activity[2]	4.37^{a}	5.79^{a}	3.66^{a}	4.06^{a}	3.41^{a}	1.30^{a}	2.67

[1] Prefix used to represent these animal groups discussed in the text.

[2] Hepatic histidine ammonia-lyase (*EC* 4.3.1.3) specific activity (mmol urocanic acid min^{-1} mg^{-1} protein).

3.5 DISCUSSION.

The primary objective of this work was to establish whether the histamine receptor antagonist, cyproheptadine significantly increased the voluntary food intake of growing or lactating rats offered a low protein : energy ratio diet over a 12 day period. The results showed that lactating rats injected with the drug, and offered a low protein concentration diet, did show a significant, positive response in daily DM intake over the first 8 days of lactation when compared to similar rats offered the same diet but injected with physiological saline. However, growing rats offered a low protein concentration diet and injected with the drug did not show an increase in daily DM intake when compared to similar rats offered the same diet but injected with saline. These results with weanling rats differ from those reported by Mercer *et al.*, (1994). Work involving lactating animals and the growing animal over a longer period of time has not previously been reported.

Impaired lactational performance as indicated by the daily litter weight gain of standardised litters, and loss of body weight resulting from the suppression of voluntary food intake with the low protein / energy ratio diet (group MLS) was similar to Trial 1, presented in Chapter 2, and also the work of Pine *et al.* (1994a,b,c) in rats and of Mahan and Mangan (1975) in pigs. However the mean food DM intakes for this group on days 10, 11 and 12 were significantly ($P<0.05$) higher when compared to those intakes for similar rats offered the same diet in Trial 1. No definitive explanation for this increase in voluntary food intake towards the end of the experimental period can be offered as DM intakes of rats from groups MHS were comparable to those in Trial 1 and also to the work of Pine *et al.* (1994a,b,c) using this diet which would suggest that the injection procedure was not having an effect on the animals. Although body weight changes and litter weight gains for group MLS were comparable with those from Trial 1, presented in Chapter 2, on an individual animal basis they did fluctuate in a manner consistent with DM intake indicating that the intakes were genuine.

The high protein concentration diet (H) was formulated to provide adequate protein to allow dams to suckle a litter of 12 pups without catabolism of significant

quantities of endogenous protein (Pine *et al.*, 1994a). Plasma histidine concentration, relative to other amino acids, would therefore not be expected to rise in these two groups and the antihistamine drug would not be expected to have any effect on DM intake, in group MHD when compared to those rats receiving saline (MHS). These results (Figure 3.1) confirm this view. However, diet L was formulated to maximise maternal body protein catabolism during lactation while still enabling a low but positive rate of litter gain (Pine *et al.*, 1994a). Pine *et al.* (1994d) previously showed that lactating rats offered this diet rapidly lost muscle protein through degradation and the bulk of the loss was achieved by day 8 of lactation, with little change occurring between day 8 and 12. This would suggest that plasma histidine concentration would be raised during the first week of lactation and therefore so would brain histamine concentration. Rats which were offered diet L and received daily injections of saline (MLS) in this study showed the characteristic suppression in food intake, (Figure 3.1) however those rats which were offered the same diet and received cyproheptadine showed a significant increase in daily DM intake when compared to group MHS until day 8 of lactation when the intakes of this group began to fall and the intakes of group MLS began to rise. The food intake patterns of these two groups (MLS and MLD) and the known body protein degradation patterns previously determined by Pine *et al.* (1994d) would appear to confirm the theory proposed by Mercer *et al.* (1994). If maternal labile body protein reserves are deplete by day 8, plasma histidine concentration would be expected to fall rapidly and voluntary food intake increase. Food intakes of rats offered diet L (group MLS) more than doubled from day 7 to day 12. This pattern of increase in DM intake was noted in the earlier experiment (Chapter 2) however, as previously mentioned, the mean increase in DM intake observed in this group at this time is larger than previously noted. There was, however a high degree of variation in food intakes between individuals in group MLS on these 3 days which may suggest that plasma histidine concentration was also variable in this group although we did not measure it. Speculatively this might reflect variable rates of labile maternal protein degradation or variable plasma histidine half lives. Litter growth rate throughout the 12 day period reflected the lactational performance of the dams during this period. The histamine antagonist,

cyproheptadine had no significant affect on the growth rate of standardised litters of dams offered diet H; both groups (MHS and MHD) had similar daily litter growth rates throughout the experimental period. However the drug did have a significant effect on lactating rats offered diet L, when compared to those receiving saline, with standardised litters of pups from dams in group MLS gaining significantly less than litters from dams in group MLD on day 6. However by day 12 there was no significant difference between daily growth rates. This pattern of pup growth for the four lactation treatment groups reflects the different patterns of voluntary food DM intake (Figures 3.1 and 3.3). Cumulative DM food intake of group MLD follows that of both groups MHS and MHD for the first 7 days of lactation, (Figure 3.1) however the cumulative litter weight gain of group MLD was only intermediate between the two groups offered the high protein diet (MHS and MHD) and group MHS. This must be a reflection of the dietary protein concentration; dams in group MLD ate the same dietary mass and therefore also an equivalent gross energy but approximately 12% less dietary protein.

Cyproheptadine had no positive effect on food DM intakes at any time over the 12 day experimental period of any of the three diets in the weanling rats. This was surprising as similar weanling rats offered a 4% protein diet in the experiment of Mercer *et al.* (1994) showed significant increases in voluntary food intake when injected daily with cyproheptadine and other histamine receptor antagonists, over a five day injection period. However, the control animals of Mercer *et al.* (1994), when offered a 4% casein diet and injected daily with saline lost 12.99 (SEM 0.93) g over the five day period reported, whereas the rats in these trials of similar weight range and treatment (WVS) lost only 7.21 (SEM 0.42) g over the same period. Although the gross energy content of their diets is not known, the excessive weight loss may imply that the diet used by Mercer *et al.* (1994) was imbalanced in amino acids to a greater extent than in this work. The protein source in the diet of Mercer *et al.* (1994) was casein, whereas the protein source used in this trial was casein, supplemented with *DL*-methionine (99:1 w/w) which added an extra 0.19 mg *L*-methionine per g DM food consumed. Speculatively, this protein source was amino

acid balanced and the 4% diet was not sufficiently protein deficient to initiate the histaminergic system.

Low protein diets have been shown to reduce the specific activity of the hepatic enzyme, histidine ammonia-lyase (*EC* 4.3.1.3) increasing the peripheral half-life of this amino acid, compounding the plasma histidine concentration increase (Kang-Lee and Harper, 1977; Clemens *et al.* 1984). The activity of this enzyme was significantly reduced in the control lactating rats offered diet L and receiving daily injections of saline, supporting the work of the above authors. This result is, perhaps, surprising as all animals were slaughtered on day 12, some 4 days after mobilisation of maternal endogenous protein reserves would be expected to have ceased and after daily food intake has returned to a level comparable to those offered diet H. The only explanation which may be offered is that perhaps the hepatic enzyme examined is slow to adapt to plasma histidine concentration; this would result in higher plasma histidine concentrations after day 8 of lactation and would tie in with the high variance in food intake previously noted for group MLS. Also surprising, the specific activity of this enzyme in the group offered diet L but administered daily with cyproheptadine (MLD) was not significantly suppressed. The drug, in addition to being a histamine receptor antagonist perhaps also inhibits suppression of the enzyme histidine ammonia-lyase.

The specific activity of enzyme histidine ammonia-lyase was not significantly altered by dietary or drug treatment in the weanling animals. This is not surprising if the very low protein diet (V) was not of sufficiently low protein concentration to necessitate the need for body protein catabolism. However, it should be remembered that the specific activity of the enzyme is expressed per mg hepatic protein, and as total hepatic protein was significantly reduced as dietary protein concentration is reduced then the peripheral half-life of histidine will also be increased as protein concentration decreases, although the drug will still exert no effect.

In summary it can be concluded that rats in early lactation offered a diet with a low protein to energy ratio reduced food DM intake. This suppression in food intake could be lifted, at least in part, by the daily administration of the strong histamine receptor antagonist, cyproheptadine for the first 8 days of lactation which supports

the suggestion that histamine plays a role in the control of food intake for low protein to energy ratio feeds. Cyproheptadine had no significant effect on the variables measured in animals offered a diet with a protein concentration sufficiently high so as not to initiate body protein degradation.

CHAPTER 4

TRIAL 3

Mammary Sensitivity to Protein and Energy Intakes During Lactation

4.1 ABSTRACT

Responses of mammary development and milk composition to changes in dietary protein and energy intake were investigated. During gestation female multiparous Sprague-Dawley rats were offered a high protein concentration diet (H; 215g crude protein (N×6.25; CP)/kg dry matter (DM)) *ad libitum* until parturition; litters were standardised to 12 pups. During lactation one group (H) were offered diet H *ad libitum* until day 10 and the remainder were offered a low protein concentration diet (L; 90g CP/kg DM) *ad libitum* until day 5, after which they received a fixed mass of one of 9 dietary regimes in order to provide a wide range of known CP and non-crude protein gross energy (NCPGE) intakes. On day 1, 5, and 10 of lactation rats from each group ($n \geq 3$) were used to estimate mammary dry mass, DNA, RNA, protein and the activity of lactose synthetase enzyme (*EC* 2.4.1.22). In addition, milk was removed and analysed for total protein, lactose and total lipid concentrations. Mammary glands of rats offered a low protein diet (L) for the first five days of lactation lost DM but showed no change in total DNA, RNA or protein. The activity of the mammary enzyme lactose synthetase was unchanged over this period, as was milk protein, lipid and lactose. Increasing NCPGE, but not crude protein intake increased mammary DM, total DNA, and protein over the 10 day period, however their was no clear pattern in milk composition during this period. Lactose synthetase activity significantly increased between day 5 and 10 in all groups, except the 2 provided with the least dietary energy and protein whose activities were unchanged. Lactational performance, as measured by daily weight gain of a standardised litter increased as both NCPGE and CP intake increased. These results show that the mammary underdevelopment shown by rats that were protein restricted during early lactation may be reversed by increasing NCPGE, however overall lactational performance depends on the intake of both energy and protein.

4.2 INTRODUCTION

Substantial development of the mammary gland can occur during early lactation in the well nourished rat (Griffith and Turner, 1961; Chapter 2). Trial 1 demonstrated the importance of adequate nutrition for this development to occur. Restricting intake through offering a diet with a low protein to energy ratio resulted in no further development post parturition (Chapter 2). However, the gland retained an ability to develop when a diet with a high protein to energy ratio was subsequently offered at time points up to mid lactation. In such cases there was an immediate increase in intake, resulting in an improved supply of both protein and energy; it was not possible to determine whether it was the improvement in protein or energy nutrition that led to mammary development (Chapter 2).

Lactational performance is a product of both milk yield and composition. Numerous experiments have provided information on the composition of rat milk and the changes associated with stage of lactation (Crnic and Chase, 1978; Grimble, 1981; Pine *et al.*, 1994c). However the conditions which influence such changes during lactation still remain unclear. Changes in milk composition associated with dietary protein restriction have been investigated at one sample point in lactation (Crnic and Chase, 1978; Grimble, 1981) and also over the first 12 days of lactation (Pine *et al.*, 1994c). While these studies show that milk protein concentration is reduced in response to dietary protein restriction, it is not clear whether this response is due to dietary protein restriction or to the associated suppression of food and hence energy intake.

The objectives of the current study were to separate the effects of re-alimentation with dietary protein (CP) and non-crude protein gross energy (NCPGE) on mammary development and milk composition after severe protein restriction for the first 5 days of lactation in the laboratory rat.

4.3 MATERIALS AND METHODS

4.3.1 Experimental Protocol

Eighty-three multiparous (second parity) female Sprague-Dawley rats (B & K Universal Ltd., Hull, U.K.) weighing on average 306 (SEM 3.14) g were housed in a room regulated at 22 °C with relative humidity between 50 - 65 % and with a light period from 06:30 - 18:30 h for a minimum of two weeks before breeding. During this period they were offered a standard rat chow (B & K Universal Ltd., Hull, U.K.) *ad libitum*. Females were placed individually in a wire bottomed cage with a proven male breeder for mating. Day 1 of gestation was the morning in which mating was confirmed through the presence of a vaginal plug, after which the females were caged individually in solid-bottomed, plastic cages for the remainder of the experiment.

From day 1 of gestation until parturition females were offered *ad libitum* a high protein concentration diet (H; 215 g crude protein (N×6.25; CP)/kg dry matter (DM)), formulated to meet the requirements of the National Research Council (1978) for vitamins and minerals. On the morning of parturition, designated day 1 of lactation, litters were standardised to 12 pups to ensure a large and uniform lactational demand. Females whose litters could not be standardised to 12 pups by cross fostering with pups born on the same morning were removed from the trial.

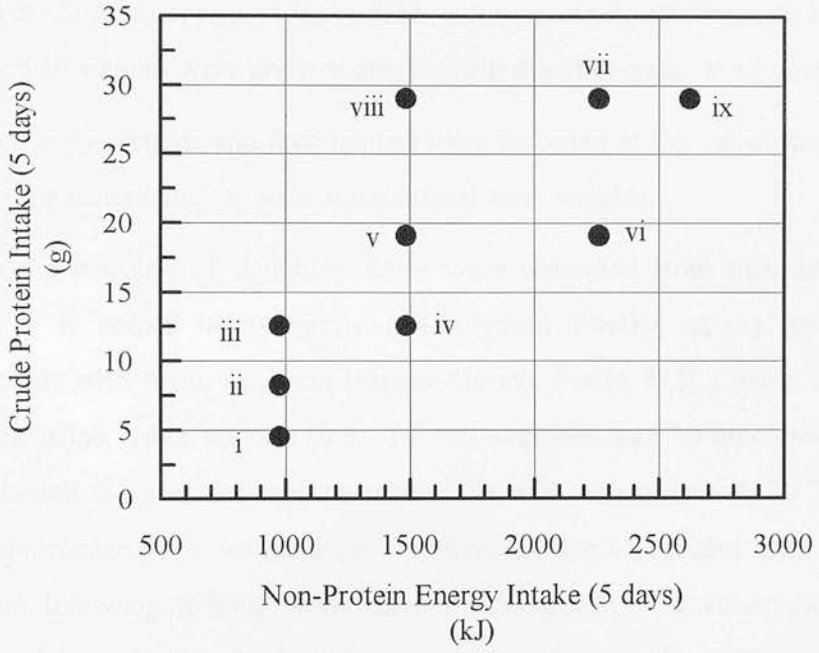

Figure 4.1 Cumulated crude protein intakes (CP; g) and non-protein gross energy (NCPGE; kJ) intakes between day 5 and 10 of lactation for dams in groups *i* (*n3*); *ii* (*n3*); *iii* (*n6*); *iv* (*n6*); *v* (*n6*); *vi* (*n6*); *vii* (*n6*); *viii* (*n6*); *ix* (*n6*). Refer to Table 4.1 and the text for further details concerning the diet.

Lactational dietary treatments consisted of one control group (H; n=4) being offered diet H, *ad libitum* until day 10 of lactation. The remaining dams were offered a low protein concentration (L; 90 g CP/kg DM) diet *ad libitum*, for the initial 4 days of lactation and on day 5 they were offered a predetermined mass of one of nine diets (*i, ii, iii, iv, v, vi, vii, viii, ix*) so as to provide nine distinct crude protein and non-crude protein gross energy intakes over the ensuing 5 days (Figure 4.1). Any rat in groups *i-ix* that did not complete their food ration over a 24 h period were subsequently offered their new ration plus the mass of the previous days refusal as fresh food so that the total intakes of food over the experimental period were as planned. Any rats that had net refusals of food at the end of this 5 day period were removed from the experiment and the data discarded. Mineral and vitamin inclusions for all groups were such as to satisfy the requirements of the National Research Council (1978), and for diets *i* - *ix* they were included so that the daily mineral and vitamin intakes for all rats was equal (Table 4.1). All diets were formulated to

provide 21.3 MJ gross energy (GE)/kg DM with a constant carbohydrate to fat value of 2.3:1, and all animals were given access to fresh drinking water at all times.

Dam body weights and feed intakes were recorded at the same time each day throughout the experiment, as were standardised litter weights.

On the morning of slaughter, dams were separated from their litters for a period of 2 h before being lightly anaesthetised (diethyl ether) and injected subcutaneously with 5 i.u. oxytocin (Sigma-Aldrich, Poole, U.K.) made up in 0.15 mol per litre saline. Milk samples (0.5 - 1.5 ml) were obtained 10 min later by gently stripping the left thoracic and inguinal teats. The milk was stored at -20 °C prior to analysis, approximately 6 weeks later. Individual dams provided only one milk sample, and following milking were killed by decapitation on either day 1(*n=6*), 5(*n=6*), or 10 (*i; n*=3, *ii; n*=3, *iii; n*=6, *iv; n*=6, *v; n*=6, *vi; n*=6, *vii n*=6, *viii n*=6, *ix; n*=6; H; *n*=4) when a complete mastectomy was performed.

4.3.2 Mammary Analysis

As the left mammary gland had been milked, no analysis was performed on this tissue.

Rates of lactose synthetase activity (*E.C. 2.1.4.1.22*) were determined in vitro, based on the method described by Vonderhaar (1977) using fresh tissue obtained from the right mammary gland.

Mammary dry mass was determined by freeze drying the right mammary gland to constant mass and multiplying by two, to account for the left side. The freeze dried mammary tissue was kept at -20 °C for approximately 1 week before being cooled in liquid nitrogen, ground to a fine powder using a pestle and mortar and analysed for total protein, DNA and RNA as described in Chapter 2.

Cell number was calculated from total mammary DNA (DNA_t) using the equation of Winick and Noble (1965):

$$\text{Cell Number} = \frac{DNA_t \text{ (mg)}}{6.2 \times 10^{-9}}.$$

Table 4.1 Composition of diets H, L and *i* - *ix*. (g/kg dry matter (DM)).

Diet	H	L	*i*	*ii*	*iii*	*iv*	*v*	*vi*	*vii*	*viii*	*ix*
Casein *	215	90	90	150	215	150	215	150	215	308	178
Maize Oil	192	230	244	226	213	226	213	226	213	186	235
Starch-sucrose †	444	530	545	505	477	505	477	505	477	416	528
Vitamin mix ‡	50	50	120	110	104	72	68	48	45	60	42
Mineral mix‡	50	50	120	110	104	72	68	48	45	60	42
Cornflour	40	40	0	0	0	0	0	0	0	0	0
Choline chloride	7	7	7	7	7	7	7	7	7	7	7
Antioxidant §	0.013	0.013	0.013	0.013	0.013	0.013	0.013	0.013	0.013	0.013	0.013
Emulsifier ¦	2	2	2	2	2	2	2	2	2	2	2

* Supplemented with DL-methionine (99:1 w/w)
† Starch and sucrose mixture (2:1 w/w)
‡ Mineral and vitamin mixtures were formulated to meet National Research Council requirements (3)
§ Butylated hydroxytoluene (g/kg fresh weight)
¦ Egg Lecithin (g/kg fresh weight)

Diet Analysis: Protein (g CP/kg DM) H 213; L 91; *i* 89; *ii* 152; *iii* 213; *iv* 151; *v* 214; *vi* 150; *vii* 215; *viii* 309; *ix* 177
Gross Energy (MJ/kg DM) H 21.3; L 21.2; *i* 21.2; *ii* 21.3; *iii* 21.3; *iv* 21.2; *v* 21.4; *vi* 21.3; *vii* 21.2; *viii* 21.2; *ix* 21.3

4.3.3 Analysis of Milk Composition

All samples were analysed individually for their protein, lactose and lipid content. Concentration of milk lactose was determined enzymatically using a lactose test kit (Boehringer Mannheim, Lewes, Sussex, U.K) after the milk had been proteinised using trichloroacetic acid (10% w/v). Total milk protein was measured by the method of Lowry *et al.* (1951), using milk casein as a standard. Milk lipid was measured gravimetrically following a three times extraction with chloroform : methanol mix (2:1 v/v) (Bligh and Dyer, 1951).

4.3.4 Statistical Analysis

The weight changes of both the dams and their litters, in addition to the data for milk composition were analysed by two way analysis of variance, with dam day 1 gestation-body weight as a covariate (Genstat5 ver.3.1) and calculation of least significant differences; *t* tests were used to compare sample means between dietary treatment groups. Multiple linear regression was used for the data from groups *i-ix* in order to differentiate the effects of both crude protein and non-crude protein gross energy intake on the variables studied (Genstat5 ver.3.1), *t* tests were used to compare regression coefficients. The following model was used:

$$\hat{Y} = \alpha + \beta gwt + \chi CP + \delta NCPGE + \varepsilon int$$

where $\hat{Y}$ is the predicted value,
α, β, χ, δ, ε are regression coefficients
gwt is day 1 maternal gestation weight (g)
CP is crude protein intake (g/5 d)
NCPGE is non-crude protein gross energy intake (kJ/5 d)
int is the interaction term calculated as:

int=(CP-mean(CP))*(NCPGE-mean(NCPGE))

4.4 RESULTS

4.4.1 Maternal body weight changes, feed intakes and litter-weight gains

Dams offered diet H *ad libitum* (H) showed no significant body weight change over the 10 day period and increased food DM intake from 13.7 (SEM 1.19) g on day 2 to 45 (SEM 1.72) g on day 10. Daily litter weight gain of this group also ($P<0.01$) increased from 12.3 (SEM 1.12) g on day 2 to 27.7 (SEM 2.30) g on day 10 ($P<0.001$). Offering diet L *ad libitum* between day 1 and 5 tended to reduce food DM intakes and increase maternal body weight loss, but not significantly so. However, lactational performance as measured by litter weight gain was reduced ($P<0.05$) when compared to rats offered diet H *ad libitum*. There were no significant differences between groups offered diet L between day 1 and 5.

Maternal body weight changes and litter weight gains for the lactation groups *i-ix* between day 5 and 10 of lactation are summarised in Table 4.2. Both NCPGE ($P<0.001$) and crude protein ($P=0.014$) intake had a positive and significant effect on maternal body weight change between day 5 and 10 of lactation. Lactational performance between day 5 and 10 was improved by increasing both NCPGE and CP intake ($P<0.001$); Table 4.2.

Table 4.2 Effects of fixed allowances of crude protein (CP; g/5 d) and non-crude protein gross energy (NCPGE; kJ/5 d) between day 5 and 10 of lactation (groups *i* - *ix*) after a period of protein restriction on dam weight change (g/5 d) and weight change of standardised litters (g/5 d). Group means, using day 1 gestation weight as a covariate. Coefficients, standard errors and probabilities of these data following multivariate regression analysis. The following model was used: $\hat{Y} = \alpha + \beta gwt + \chi CP + \delta NCPGE + \varepsilon int$, where gwt is day 1 maternal gestation weight (g); CP is total 5 day crude protein intake (g); NCPGE is the total 5 day non-crude protein gross energy intake (kJ) and int the interactive term calculated as int=(CP-mean(CP))*(NCPGE-mean(NCPGE)).

	Mean Data						Model		
NCPGE / CP	**975**	**1485**	**2260**	**2625**	**Pooled SED**		**Adj. r^2 (%)**	**Estimate**	**SE**	***P***
Dam Weight Change (g/5 d)							79.6			<0.001
4.55	-48.4					α		-89.2	21.4	<0.001
8.25	-52.4					β		0.0034	0.067	0.961
12.55	-47.0	-29.4			6.30	χ		0.801	0.314	0.014
19.1		-21.4	-12.2			δ		0.029	0.004	<0.001
29.05		-26.3	5.7	21.1		ε		0.0008	0.000	0.653
Litter Weight Change (g/5 d)							90.4			<0.001
4.55	24.3					α		-14.1	14.9	0.350
8.25	36.7					β		0.067	0.047	0.162
12.55	51.5	53.5			5.50	χ		1.975	0.218	<0.001
19.1		69.4	80.9			δ		0.016	0.003	<0.001
29.05		85.0	100.3	104.4		ε		0.000	0.000	0.653

4.4.2 *Effect of dietary treatment on mammary gland composition*

Offering diet H *ad libitum* throughout the 10 day experimental period led to a significant ($P<0.01$) loss of mammary DM but significant ($P<0.05$) increases in the total content of mammary DNA, RNA, and protein; Table 4.3.

Table 4.3 Mammary composition and lactose synthetase activity of rats slaughtered on day 1 of lactation and from rats offered a low (L)- protein concentration diet *ad libitum* between day 1 and 5 of lactation and also from rats slaughtered on day 10 and offered a high (H)- protein concentration diet (group H) *ad libitum* for the experimental period.

Diet		**Diet L**	**Diet H**	
Day of Slaughter	**Day 1**	**Day 5**	**Day 10**	**Pooled**
n **. . . .**	**6**	**6**	**4**	**SED**
DM (g)	12.21^{b}	8.75^{a}	9.15^{a}	0.94
DNA_t (mg)	$12.68^{a,b}$	11.40^{b}	18.50^{a}	3.02
RNA_t (mg)	59.7^{b}	86.8^{b}	133.3^{a}	15.65
Total Protein (g)	2.42^{b}	2.34^{b}	3.63^{a}	0.34
Lactose Synthetase Activity[†]	53.0^{a}	49.1^{a}	245.6^{b}	20.27

[a,b] Means within the same row with different superscript differ ($P<0.05$)
[†] Lactose Synthetase Activity (*n*moles lactose formed mg^{-1} DNA min^{-1})

Mammary dry mass declined ($P<0.05$) from 12.21 to 8.75 g between day 1 and day 5 of lactation in dams offered diet L, whereas there were no significant changes in mammary protein, total mammary DNA (DNA_t) or total mammary RNA (RNA_t) in these rats over this period (Table 4.3).

Table 4.4 Coefficients, standard errors and probabilities for mammary dry mass (DM), total mammary DNA (DNA_t), and total mammary protein following multivariate regression using the data from rats offered fixed allowances of crude protein (CP; g/5 d) and non-crude protein gross energy (NCPGE; kJ/5 d), groups *i-ix*, betwen day 5 and 10 after a period of protein restriction. The following model was used: $\hat{Y} = \alpha + \beta gwt + \chi CP + \delta NCPGE + \varepsilon int$, where gwt is day 1 maternal gestation weight (g); CP is total 5 day crude protein intake (g); NCPGE is the total 5 day non-crude protein gross energy intake (kJ) and int the interactive term calculated as int=(CP-mean(CP))*(NCPGE-mean(NCPGE)).

	Adj. r^2 (%)	Estimate	SE	*P*
DM (g)	57.8			<0.001
α		-3.93	2.72	0.156
β		0.017	0.009	0.056
χ		0.048	0.039	0.225
δ		0.002	0.001	<0.001
ε		0.000	0.000	0.198
DNA_t (mg)	20.6			0.009
α		3.40	11.00	0.757
β		0.007	0.035	0.844
χ		-0.057	0.158	0.719
δ		0.006	0.002	0.010
ε		0.000	0.000	0.395
RNA_t (mg)	53.6			<0.001
α		-27.1	56.6	0.634
β		.155	0.180	0.395
χ		1.731	0.814	0.040
δ		0.029	0.012	0.015
ε		0.003	0.001	0.010
Total Protein (g)	53.8			<0.001
α		-0.810	1.08	0.454
β		0.006	0.003	0.086
χ		0.025	0.016	0.116
δ		0.001	0.000	0.003
ε		0.000	0.000	0.895

Table 4.5 Effects of fixed allowances of crude protein (CP; g/5 d) and non-crude protein gross energy (NCPGE; kJ/5 d) between day 5 and 10 of lactation (groups *i-ix*) on mammary composition after a period of protein restriction on mammary dry mass (g).

NCPGE / CP	975	1485	2260	2625	Pooled SED
DM (g)					
4.55	4.47^{b}				
8.25	3.97^{b}				
12.55	4.42^{b}	5.44^{b}			0.94
19.1		5.95^{b}	6.61^{b}		
29.05		5.69^{b}	$7.47^{a,b}$	9.28^{a}	
DNA_t (mg)					
4.55	8.04^{a}				
8.25	7.97^{a}				
12.55	$10.95^{a,b}$	$14.90^{a,b}$			3.02
19.1		$14.17^{a,b}$	$17.42^{b,c}$		
29.05		$13.86^{a,b}$	$13.48^{a,b}$	20.67^{c}	
RNA_t (mg)					
4.55	75.4^{c}				
8.25	83.0^{c}				
12.55	99.3^{c}	91.0^{c}			15.65
19.1		102.9^{c}	$118.0^{b,c}$		
29.05		$104.8^{b,c}$	142.5^{b}	185.1^{a}	
Total Protein (g)					
4.55	1.82^{c}				
8.25	$1.89^{b,c}$				
12.55	$2.20^{b,c}$	$2.42^{b,c}$			0.34
19.1		$2.47^{b,c}$	3.14^{b}		
29.05		$2.89^{b,c}$	3.15^{b}	3.77^{a}	
RNA_t : DNA_t					
4.55	9.11				
8.25	10.81				
12.55	10.42	8.12			2.56
19.1		9.15	7.53		
29.05		9.22	12.73	10.60	
Protein : DNA_t					
4.55	222				
8.25	250				
12.55	235	200			70.4
19.1		217	247		
29.05		266	296	207	

^{a,b} Means within the same variable with different superscript differ ($P < 0.05$)

The effects of NCPGE and CP intake on mammary development after five days severe protein restriction are shown in Tables 4.4 and 4.5. Dietary protein had no significant effect on mammary dry mass (P=0.225), DNA_t (P=0.719), or total mammary protein (P=0.116), however RNA_t, was (P=0.04) effected by CP intake. In contrast, mammary dry mass (P<0.001), DNA_t (P=0.01), total mammary protein (P=0.003), and RNA_t (P=0.015) all increased as NCPGE intake increased after the period of protein restriction.

4.4.3 Mammary cell activity

The activity of the mammary enzyme lactose synthetase was increased (P<0.001) between day 1 and 10 in rats from group H, however, there was no significant difference between day 1 and 5 for rats offered diet L (Table 4.3).

Table 4.6 Effects of fixed allowances of crude protein (CP; g/5 d) and non-crude protein gross energy (NCPGE; kJ/5 d) after a period of protein restriction on lactose synthetase activity (*n*moles lactose formed mg^{-1} DNA min^{-1}). Means, using day 1 gestation weight as a covariate, with different letter superscripts are significantly different (P<0.05).

NCPGE / CP	975	1485	2260	2625	SED
4.55	61.6^{e}				
8.25	63.2^{e}				
12.55	152.9^{d}	$180.5^{c,d}$			20.27
19.1		$205.5^{b,c}$	$167.5^{c,d}$		
29.05		148.1^{d}	265.4^{a}	$229.5^{a,b}$	

Lactose synthetase activities for groups *i-ix* are shown in Table 4.6 and the regression analysis in Table 4.7. Offering diets of very low protein and energy, groups *i* and *ii* resulted in no significant change in activity on day 10 when compared to those on day 5. Lactose synthetase activity increased in all other groups when

compared to the enzyme activities on day 5 and the regression analysis shows this response to be related to NCPGE intake (P=0.034) and not CP intake (P=0.112).

Table 4.7 Coefficients, standard errors and probabilities for lactose synthetase activity (*n*moles lactose formed mg^{-1} DNA min^{-1}) following multivariate regression using the data from rats offered fixed allowances of crude protein (CP; g/5 d) and non-crude protein gross energy (NCPGE; kJ/5 d), groups *i-ix*, betwen day 5 and 10 after a period of protein restriction. The following model was used: $\hat{Y} = \alpha + \beta gwt + \chi CP + \delta NCPGE + \varepsilon int$, where gwt is day 1 maternal gestation weight (g); CP is total 5 day crude protein intake (g); NCPGE is the total 5 day non-crude protein gross energy intake (kJ) and int the interactive term calculated as int=(CP-mean(CP))*(NCPGE-mean(NCPGE)).

	Adj. r^2 (%)	Estimate	SE	*P*
Lactose synthetase activity	39.8			<0.001
α		25.0	105.0	0.813
β		0.082	0.336	0.807
χ		2.46	1.520	0.112
δ		0.047	0.022	0.034
ε		0.000	0.002	0.783

† day 1 maternal gestation weight

4.4.4 Milk Composition

The effects of lactational dietary treatment on milk composition are summarised in Table 4.8 and 4.9. The milk lactose concentration of rats offered diet H *ad libitum* increased (P<0.001) between day 1 and 10, whereas the total milk lipid concentration (P<0.05) decreased and the total milk protein concentration was unchanged (Table 4.8). Milk lactose, protein and lipid concentrations of rats offered diet L were unchanged between day 1 and 5 of lactation (Table 4.8). On day 10, milk lactose concentration tended to be higher than on both day 1 and day 5, however no clear trend was observed between groups offered Diets *i-ix* (Table 4.9).

Table 4.8 Milk composition of milk from rats slaughtered on day 1 of lactation and from rats offered a low (L)- protein concentration diet *ad libitum* between day 1 and 5 of lactation and also from rats slaughtered on day 10 and offered a high (H)- protein concentration diet (group H) *ad libitum* for the experimental period.

Diet		**Diet L**	**Diet H**	
Day of Slaughter	**Day 1**	**Day 5**	**Day 10**	**Pooled**
n **....**	**6**	**6**	**4**	**SED**
Lactose (mg/g)	12.81[b]	13.56[b]	24.09[a]	2.68
Total Protein (mg/g)	88.3	75.0	88.6	9.67
Total Lipid (mg/g)	151.0[a]	166.0[a]	92.5[b]	18.74

[a,b] Means within the same row with different superscript differ ($P < 0.05$)

During the period of lactation studied, milk protein concentration was not influenced either by changing dietary CP or NCPGE intake. The milk lipid concentration of rats offered diet L tended to increase between day 1 and day 5 of lactation but not significantly so. In the re-alimentation period, milk lipid concentration ($P<0.05$) decreased with NCPGE alleviation but did not change with CP allowance.

Table 4.9 Effects of fixed allowances of crude protein (CP; g/5 d) and non-crude protein gross energy (NCPGE; kJ/5 d) intake after a period of protein restriction on the composition of milk taken on day 10 of lactation (groups *i-ix*).

NCPGE / CP	975	1485	2260	2625	Pooled SED
Lactose (mg/g)					
4.55	$22.5^{a,c}$				
8.25	$16.5^{a,b,c}$				
12.55	$16.1^{a,b}$	15.1^{b}			2.68
19.1		$21.6^{a,b,c}$	$22.9^{a,b,c}$		
29.05		$18.1^{a,b,c}$	$19.7^{a,b,c}$	22.4^{c}	
Total Protein (mg/g)					
4.55	$88.8^{a,b}$				
8.25	93.0^{a}				
12.55	89.8^{a}	$88.1^{a,b}$			9.67
19.1		70.0^{b}	$75.3^{a,b}$		
29.05		90.5^{a}	$77.4^{a,b}$	$86.0^{a,b}$	
Total Lipid (mg/g)					
4.55	176.3^{a}				
8.25	$148.0^{a,b}$				
12.55	178.1^{a}	$143.6^{a,b}$			22.95
19.1		129.8^{b}	$154.3^{a,b}$		
29.05		$155.1^{a,b}$	127.6^{b}	103.1^{b}	

^{a,b} Means within the same variable with different superscript differ ($P<0.05$)

4.5 DISCUSSION

Following Trial 1, described in Chapter 2, the primary objective of this work was to separate the effects of dietary protein and energy intakes on mammary development and lactational performance after an initial 5 day protein restriction. The previous trials presented in this thesis have shown that the food intake of lactating rats is sensitive to the protein-energy ratio of the diet. The ability to experimentally manipulate protein and energy intake seperately, was therefore constrained by the dietary ratio of protein to energy, as the rats had to eat the entire allocation to fulfill statistical criteria.

The results have shown that mammary cell number and secretory cell activity were more sensitive to energy than protein intake, and overall lactational performance responds both to increases in dietary protein and energy. Although the remarkable rapidity of this response has previously been reported (Chapter 2) the gland's sensitivity to re-alimentation with differing measures of energy and protein is both important and novel to the dairy industry.

The results for dam body weight changes, litter weight changes, mammary variables and milk composition for diet H and L agree with the previous work described in this thesis and confirm that offering a diet of low protein to energy ratio (L) drastically impairs voluntary food intake and therefore lactational performance (Pine *et al.*, 1994a,b,c).

It has been suggested that total tissue DNA content (DNA_t) may be used as an accurate indicator of cell number and therefore development (Mirsky and Ris, 1949). This was later confirmed for mammary tissue (Tucker, 1987). Using increase in mammary DNA_t as an indication of increases in secretory cell number makes the assumption that the stromal tissue cell number remains constant throughout the experimental period. This has been shown to be the case, as both adipose and connective tissue cell population is largely determined before first conception and any change in DNA_t observed during lactation may therefore be attributed to the secretory cell population (Paape and Sinha, 1971). When analysing milk to determine composition, it is essential that the milk sample obtained is similar to that generally obtained by the suckling young. Milk fat concentration in rats has been shown to be diminished through prolonged milk stasis (> 4 h) within the gland (Grigor *et al.*, 1986), and therefore milk samples were obtained after only 2 h separation of dam and litter in this study. The litters were separated from the dam at the beginning of the light period so milk samples were obtained during the early light phase to minimise the effects of diurnal variation in mammary lipogenesis and lactose synthesis (Williamson *et al.*, 1984).

Offering diet L between day 1 and 5 of lactation led to a significant ($P<0.001$) decrease (28%) in mammary dry mass, which has previously been ascribed to the loss

of mammary fat (Chapter 2). The regression analysis attributed this loss of DM to NCPGE intake which would suggest that mobilisation of mammary fat was also occuring in this experiment, although this was not determined through analysis. There was no significant change in DNA_t of rats offered diet L between day 1 and 5 and offering diets *i* and *ii* between day 5 and 10 tended to decrease total DNA but not significantly. It is not clear from this experiment whether this increase in cell mass was due to an increase in mitosis or a decrease in the rate of secretory cell turnover. These results do show that the increase in cell mass observed when rats, protein restricted during early lactation were offered a diet of high protein to energy ratio was due to the increased supply of energy yielding nutrients associated with the increase in voluntary intake and not the increase in dietary protein as previously assumed (Chapter 2). Perhaps the co-ordinated adaptations in metabolism (homeorhesis) proposed by Bauman and Currie (1980), are better designed to partition available amino acids rather than other energy supplying nutrients towards the mammary gland. Unfortunately, it is still unknown whether the increase in energy yielding nutrients are acting directly on the gland or through other factors *e.g.* the production of a blood borne signal, whether it be hormone or metabolite to increase cell mass.

Since mammary RNA is intimately related to the biosynthesis of protein, this nucleic acid may be used as an indication of the cell's synthetic potential (Winick and Noble, 1965). As RNA is closely associated with protein synthesis it is not surprising that increasing the intake of both CP and NCPGE increased total mammary RNA content. Of perhaps more importance is the RNA : DNA ratio which remained remarkably constant over the 10 day lactational period and between dietary treatment regimes (Tables 4.3 and 4.5). This would suggest that each cell was able to retain an adequate quantity of RNA to support protein synthesis and the total mammary protein synthetic capacity was determined by mammary cell mass and substrate availability. In addition, the total mammary protein : DNA ratio was not significantly altered over the lactation period studied or between dietary groups which further suggests that the cellular protein synthesis mechanism had been spared (Tables 4.3 and 4.5).

It has previously been reported that offering low protein concentration diets will reduce milk protein concentration during mid-late lactation (Crnic and Chase, 1978; Sturnman *et al.*, 1986) and also during the first half of lactation (Pine *et al.*, 1994c). Although, the total milk protein composition results from this experiment are in very close agreement with the work of Pine *et al.*, (1994c), the values from rats offered a low protein concentration diets were not significantly lower than those from rats offered high protein concentration diets due mainly to the variability of the results arising from relatively small group sizes; indicated by the SED value (Table 4.8). Secretory cell activity, as measured by the specific activity of lactose synthetase enzyme (*EC* 2. 4.1.22) did not alter between day 1 and 5 in dams offered diet L, in agreement with trial 1 (Chapter 2). Between day 5 and 10, lactose synthetase specific activity increased markedly in all groups except *i* and *ii*. As the specific activity of the enzyme from rats offered diet *iii* which received the same NCPGE but greater CP intake was significantly higher it would suggest that the enzyme's activity is suppressed when diets of low protein to energy ratio are offered. As half the enzyme's complex comprises the milk protein α-lactalbumin, the mammary gland may be sparing either or both cellular and milk protein at the expense of lactose production when offered diets leading to severe protein deficiency. However, the regression analysis indicated that it was NCPGE and not CP intake that was having a significant effect on specific activity, and when a diet of extremely high protein to energy ratio (diet *viii*) was offered there was a significant suppression of activity. The glycoprotein α-lactalbumin and hence protein availability have been implicity associated with the regulation of lactose synthetase activity (Palmitter 1969b; Nicholas *et al.*, 1984). But this work suggests that the enzyme activity was limited by the shortage of energy yielding nutrients and not protein. Kuhn *et al.* (1980) suggested that the specific activity of this enzyme could also be constrained by the substrate glucose. This may well have been the case in this experiment when the low energy diets were offered. Using DNA_t (Table 4.5) and specific lactose synthetase activity (Table 4.6) it is possible to calculate theoretical daily lactose production rates. It is also possible to predict milk yield from these rats from the litter weight gain using the equations described by Pine *et al.* (1994c). Using this prediction and the milk lactose

concentration data (Table 4.8) we can estimate the daily lactose requirements. It is estimated that rats produce 14.2 g of milk over the first 24 h following parturition which would lead to a requirement of 0.18 g lactose, which is relatively low due to the high concentration of immunoglubulins present over this period. The estimated production rate would be 0.35 g/d which would indicate that the specific enzyme activity is not limiting production. On day 10, rats from group *ix* had an estimated milk yield of 55 g/day, requiring 1.24 g of lactose. From the specific lactose synthetase activity it may be predicted that 2.38 g would be produced, once again, a good surplus. Milk yield on day 10 for rats offered diet *i* would have been approximately 14.4 g which would lead to a lactose requirement of 0.32 g, however, predicted lactose production for this period would only be 0.3 g suggesting that suppressed intakes of energy during lactation may have contstrained the activity of the lactose synthetase complex and as a result, limited milk production. This is interesting as it has been suggested that the osmotic pressure of milk and therefore daily milk volume is determined primarily by lactose and the diffusible ion concentration (Palmiter, 1969a; Pine *et al.*, 1994c). Lactose synthetase activity could be limiting daily milk volume when diets of low protein to energy ratio are offered to lactating rats. Although the lactose synthetase assay was carried out in vitro with an excess of substrate present which may lead to results different from the *in vivo* situation, the consistency of the milk lactose concentration between dietary treatment groups would suggest that the animal partitions sufficient substrate for lactose production to prevent a deficiency occurring except under the most severe dietary restrictions.

Total milk lipid concentration decreased in lactating rats which were re-alimented with diets of favourable protein to energy ratio, in agreement with Pine *et al.* (1994c). However, due to a vastly increased (at least 250%) milk production in these animals at this time, actual lipid intake of their litters would still be in excess of their contemporaries receiving milk from severely protein restricted animals. Suggestions for the increased milk fat concentration of milk produced by protein restricted rats have been discussed previously (Pine *et al.*, 1994c).

In summary, it can be concluded that when rats are subjected to a period of severe protein deficiency during early lactation food dry matter intake and mammary gland development are compromised and, in addition milk composition is significantly altered. However, increasing the supply of NCPGE leads to a rapid increase in secretory cell number and secretory cell activity. Observed increases in overall lactational performance, measured by the weight gain of a standardised litter depended on both NCPGE and CP intake, but it would appear that NCPGE is more influential in allowing recovery from previous protein to energy malnutrition than protein alone.

CHAPTER 5

TRIAL 3

Sensitivity of Body Tissues to Protein and Energy Intakes During Lactation

5.1 ABSTRACT

The effects of dietary protein and energy intake following severe protein restriction post-partum on tissue protein and fat reserves was studied in rats. During gestation female, multiparous Sprague-Dawley rats were offered a high protein concentration diet (H; 215g crude protein (N×6.25; CP)/kg dry matter (DM)) *ad libitum* until parturition when litters were standardised to 12 pups. During lactation one group (H; n=4) was offered diet H *ad libitum* until day 10 and the remainder were offered a low protein concentration diet (L; 90g CP/kg DM) *ad libitum* until day 5, after which they received a fixed mass of one of 9 dietary regimes in order to provide a wide range of known CP and non-crude protein gross energy (NCPGE) intakes. On day 1, 5, and 10 of lactation rats from each group (n≥3) were used to estimate dry mass, crude protein, fat and ash composition of the carcass, abdominal and thoracic contents (organs) and skin.

Carcass and skin CP was not mobilised over the first 5 days of lactation, after which offering low CP diets following protein restriction significantly reduced carcass, organ and skin protein content, whereas offering high CP diets resulted in no change. Body fat was mobilised throughout the 10 day period, irrespective of dietary treatment, however offering low NCPGE diets tended to increase body fat loss.

5.2 INTRODUCTION

The concept that animals supplement their dietary intake of protein and energy by catabolism of labile body stores during times of stress, is widely documented for rats (Allison and Wannemacher, 1965; Cherel *et al.*, 1991), cattle (Pacquay *et al.*, 1972; Bauman and Currie, 1980) and chicks (Fisher *et al.*, 1964). Lactation, and in particular early lactation, can impose an enormous demand on the mother for both energy and protein. Although there is often, after parturition, a considerable increase in food intake when diets of high quality are offered, the use of both body fat and protein stores is commonplace in rats (Naismith *et al.*, 1982, Pine *et al.*, 1994a,b,c), goats (Pacquay *et al.*, 1972; Barnes and Brown, 1990), cattle (Bauman and Currie, 1980) and humans (Butte *et al.*, 1984). If a diet of low protein concentration is offered during lactation, food intake is rapidly suppressed in rats (Naismith *et al.*, 1982; Pine *et al.*, 1994a,b,c; Chapters 2 and 3) and also in pigs (Mahan and Mangan, 1975). Under these circumstances the demand on maternal body reserves is dramatically increased. The maximum mass of body protein available for lactation appears to be between 200 to 250 g/kg for both rats (Allison and Wannemacher, 1965; Pine *et al.*, 1994a) and cows (Botts *et al.*, 1979); the primary site of protein mobilisation is reported to be the skeletal muscle (Swick and Benevenga, 1977; Bryant and Smith, 1982; Millican *et al.*, 1987) and the secondary site the skin (Cherel *et al.*, 1991). The pattern of protein loss in the gastrocnemius muscle of the rat has been shown to be non-linear, with the highest rates of loss occurring during the first 4 days of lactation (Pine *et al.*, 1994d), and complete depletion of labile protein reserves by day 8. Although endogenous protein catabolism is increased when protein-energy imbalanced diets are offered, it is not known if the mechanism is sensitive to dietary energy or protein intake.

Animals, when possible, lay down stores of fat during gestation to meet the demands of lactation; this is thought to be under the hormonal control of progesterone (Garnsworthy, 1988). However, the fatness of cows at parturition, measured by condition scoring has been reported to affect subsequent nutrition (Garnsworthy and Topps, 1982). Females with a lower condition score at calving,

compensate for their lack of fatness by increasing their food intake, thereby maintaining lactational performance at a level comparable to their fatter contemporaries (Garnsworthy, 1988). It may therefore be assumed that mobilisation of fat during lactation is not dependent upon lactational performance and the rate of its depletion is under hormonal control (Naismith *et al.*, 1982; Pine *et al.*, 1994a). It has also been shown that the hormonal milieu during early lactation favours fat mobilisation and prevents lipid synthesis (Marinchenko *et al.*, 1992); increasing the supply of energy available for lactation.

The primary objective of this work was to separate the effects of protein and energy intake on maternal body reserves during lactation after a 5 day period of severe protein restriction.

5.3 MATERIALS AND METHODS

5.3.1 Experimental Protocol

The animals in this study were also used in the investigation of protein and energy re-alimentation on changes in mammary development and milk composition; the experimental design has been fully described in Chapter 4, as have the results. Briefly, eighty-three multiparous female Sprague-Dawley rats were offered a high protein diet (H; 215g crude protein (N×6.25; CP)/kg dry matter (DM)) *ad libitum* from conception to parturition when litters were standardised to 12 pups. The control group (H; n=4) continued to receive diet H *ad libitum* for a further 10 days. The remainder were offered a low protein concentration diet (L; 90 g CP/kg DM) *ad libitum* for a 4 day period; on day 5 they received a predetermined mass of one of nine diets (*i, ii, iii, iv, v, vi, vii, viii, ix*) so as to provide nine distinct protein and energy intakes over the ensuing 5 days (Figure 1). Any rats which had net refusals of food over this 5 day period were removed from the trial.

Throughout the experiment, maternal body-weight and feed intakes were recorded daily as were litter weights during the 10 day lactation period. All animals were given free access to fresh drinking water at all times. On days 1, 5 and 10 of

lactation dams were lightly anaesthetised (diethyl ether) and injected subcutaneosly with 5 IU oxytocin, following a 2 h separation period from their litters, and the left thoracic and inguinal mammary teats were milked, after which they were slaughtered by decapitation along with their litters.

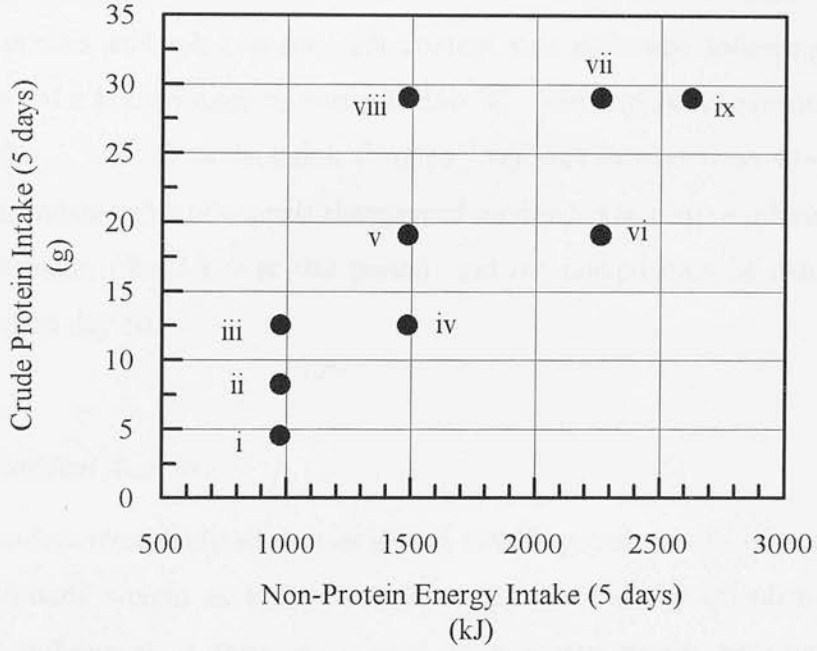

Figure 5.1 Cumulated crude protein intakes (CP; g) and non-protein gross energy (NCPGE; kJ) intakes between day 5 and 10 of lactation for dams in groups *i* (*n3*); *ii* (*n3*); *iii* (*n6*); *iv* (*n6*); *v* (*n6*); *vi* (*n6*); *vii* (*n6*); *viii* (*n6*); *ix* (*n6*).

5.3.2 Carcass Analysis

After decapitation, the skin and mammary gland of dams were dissected away and the abdominal and thoracic contents (organs) in their entirety were removed, and stored at -20 °C. The mammary gland was analysed for dry matter (DM), protein, DNA, RNA and the specific activity of lactose synthetase (*EC* 2.4.1.22) enzyme (see Chapter 4 for a full discussion of these results). The carcasses, organs and skins were analysed separately for DM, protein, ash and fat. The DM content was designated as the mass following freeze-drying to constant mass. Protein was calculated as Kjeldahl

N×6.25. Carcass and abdominal organ fat was estimated from the gross energy values using the equation of (Pine *et al.*, 1994a):

$$\text{fat (g/kg DM)} = (\text{GE} - 23.6 \times \text{CP}/1000)/39.6 \times 1000,$$

where 23.6 and 39.6 represent the GE contents (MJ/kg) of CP and fat respectively (McDonald *et al.*, 1988). Skin fat content was estimated as the DM minus the sum of the total protein and ash content. Ash content was estimated following complete combustion of a known mass of tissue at 550 °C. The carcass composition change between day 5 and 10 of lactation (groups *i-ix*) was estimated as the difference between the mean value of animals slaughtered on day 5 which were offered the same dietary treatment (diet L) over this period, and the composition of individual rats slaughtered on day 10.

5.3.3 Statistical Analysis

The data were analysed statistically by, two-way analysis of variance, with day 1 gestation-body weight as a covariate (Genstat5 ver.3.1); by calculation of least significant differences, *t* tests were used to compare means between lactation treatment groups. In addition, multiple linear regression was used for the data from groups *i-ix* in order to differentiate the effects of both crude protein and non-crude protein gross energy intake on the variables studied (Genstat5 ver.3.1), *t* tests were used to compare regression coefficients. The following model was used:

$$\hat{Y} = \alpha + \beta\text{gwt} + \chi\text{CP} + \delta\text{NCPGE} + \varepsilon\text{int}$$

where $\hat{Y}$ is the predicted value,
α, β, χ, δ, ε are regression coefficients
gwt is day 1 maternal gestation weight
CP is crude protein intake (g/5 d)
NCPGE is non-crude protein gross energy intake (kJ/5 d)
int is the interaction term calculated as:

int=(CPI-mean(CPI))*(NCPGE-mean(NCPGE))

5.4 RESULTS

The effects of dietary CP and NCPGE intake after a period of protein restriction during lactation on maternal body weight loss, and lactational performance have been previously reported in Chapter 4 and are summarised in Table 5.1. Both NCPGE and CP intake had a positive effect ($P<0.05$) on maternal body weight change and lactational performance after a period of severe protein restriction.

Table 5.1 Effects of fixed allowances of crude protein (CP; g/5 d) and non-crude protein gross energy (NCPGE; kJ/5 d) between day 5 and 10 of lactation (Groups *i* - *ix*) after a period of protein restriction on dam weight change (g/5 d) and also on the growth rate of standardised litters (g/5 d). Means, using day 1 gestation weight as a covariate, with different symbol superscripts are significantly different ($P<0.05$)

NCPGE / CP	975	1485	2260	2625	SED
Dam Weight Change (g/5 d)					
4.55	-48.4^{e}				
8.25	-52.4^{e}				
12.55	-47.0^{e}	-29.4^{d}			6.30
19.1		-21.4^{c,d}	-12.2^{c}		
29.05		-26.3^{d}	5.7^{b}	21.1^{a}	
Litter Weight Change (g/5 d)					
4.55	24.3^{e}				
8.25	36.7^{d,e}				
12.55	51.5^{d}	53.5^{d}			5.50
19.1		69.4^{b}	80.9^{b}		
29.05		85.0^{b}	100.3^{a}	104.4^{a}	

5.4.1 The effects of dietary treatment on carcass analysis.

Carcasses of animals slaughtered on day 5 and offered diet L *ad libitum* from day 1 of lactation showed no significant change in either total protein or total ash content. However the carcasses of these animals contained less fat ($P<0.05$) than the carcasses from animals slaughtered on day 1 (Table 5.2). Neither the total protein nor

ash contents of carcasses from dams offered diet H *ad libitum* (group H) for the 10 day experimental period were different from those animals slaughtered on day 1 of lactation (Table 5.2). The fat content of these carcasses was lower ($P<0.001$) than that of carcasses from animals slaughtered on day 1 (26.98 (SEM 2.93) compared with 12.38 (SEM 2.12) g) and also lower ($P<0.05$) than carcasses from dams offered diet L between day 1 and 5 of lactation (Table 5.2).

Table 5.2 Composition of carcass, abdominal and thoracic contents (organs) and skin on day 1 of lactation from rats offered a low (L)- protein concentration diet *ad libitum* between day 1 and 5 of lactation and from rats slaughtered on day 10 and offered a high (H)- protein concentration diet (group H) *ad libitum* for the experimental period.

Diet	**Diet L**		**Diet H**	
Day of Slaughter	**Day 1**	**Day 5**	**Day 10**	**Pooled**
n	**6**	**6**	**4**	**SED**
Carcass				
Protein (g)	32.42	33.10	33.21	1.71
Fat (g)	26.98[a]	19.71[b]	12.38[c]	2.91
Ash (g)	7.99	7.45	7.51	0.33
Organs				
Protein (g)	6.80[a]	5.21[b]	6.31[a]	0.43
Fat (g)	31.40[a]	27.62[a]	17.10[b]	3.66
Ash (g)	0.51	0.43	0.66	0.12
Skin				
Protein (g)	11.96	11.73	11.15	0.52
Fat[†] (g)	20.85[a]	15.42[b]	8.65[c]	2.14
Ash (g)	0.24	0.25	0.25	0.09

[a,b] Means within the same row with different superscript differ ($P < 0.05$)

[†] Skin fat composition estimated from the difference between skin DM and total skin protein and ash content.

The effects of NCPGE and CP intake on carcass composition after 5 days severe protein restriction are shown in Table 5.3 with the regression analysis shown in Table 5.4. Offering a very low CP and NCPGE ration (groups *i* and *ii*) between day 5 and 10 of lactation ($P<0.05$) reduced the total carcass crude protein content. The

regression analysis confirms that increasing the dietary CP intake decreases (P=0.048) this carcass CP loss, whereas increasing NCPGE intake does not (Table 5.4).

Table 5.3 Effects of fixed allowances of crude protein (CP; g/5 d) and non-crude protein gross energy (NCPGE; kJ/5 d) between day 5 and 10 of lactation (groups *i-ix*) on changes in carcass composition after a period of protein restriction. Values are mean gains in constituent weights between day 5 and 10 (g)

NCPGE / CP	975	1485	2260	2625	Pooled SED
Carcass Protein (g)					
4.55	-5.02[b]				
8.25	-5.06[b]				
12.55	-3.18[a,b]	-2.97[a,b]			1.76
19.1		-3.60[a,b]	-3.82[a,b]		
29.05		-0.38[a]	-3.50[a,b]	-1.20[a]	
Carcass Fat (g)					
4.55	-11.08[a]				
8.25	-11.96[a]				
12.55	-9.16[a,b]	-8.26[a,b]			2.75
19.1		-9.18[a,b]	-4.00[b]		
29.05		-8.25[a,b]	-6.71[a,b]	-5.98[a,b]	
Carcass Ash (g)					
4.55	-0.61				
8.25	-1.10				
12.55	-0.74	-0.44			0.35
19.1		-0.91	-0.79		
29.05		-0.74	-0.87	-0.71	

[a,b] Means within the same variable with different superscript differ ($P < 0.05$)

All animals lost a significant (P<0.05) mass of carcass fat between day 5 and 10 of lactation (Table 5.3), with animals on the low energy diets tending to lose the most. The regression analysis confirmed that the dietary NCPGE intake was the significant (P=0.023) component of the model, as NCPGE intake increased carcass fat loss was reduced although the value of the coefficient was small (Table 5.4). Carcass ash tended to be lower in all groups on day 10 when compared to day 5, however

there was no clear pattern to this slight reduction and the fitted regression equation was not significant.

Table 5.4 Coefficients, standard errors and probabilities for the change in crude protein (CP), and fat composition of carcasses following multivariate regression using the data from rats offered fixed allowances of crude protein (CP) and non-crude protein gross energy (NCPGE), groups *i-ix*, betwen day 5 and 10 after a period of protein restriction. The following model was used: $\hat{Y} = \alpha + \beta gwt + \chi CP + \delta NCPGE + \varepsilon int$. Where int is the interaction term calculated as: int=(CP-mean(CP))*(NCPGE-mean(NCPGE)) and gwt is day 1 maternal gestation weight.

	Adj. r^2 (%)	Estimate	SE	*P*
Carcass CP (g)	21.4			0.006
α		11.55	5.63	0.046
β		0.056	0.018	0.003
χ		0.168	0.083	0.048
δ		-0.001	0.001	0.395
ε		0.000	0.000	0.873
Carcass Fat (g)	12.7			0.043
α		-0.46	8.51	0.957
β		0.026	0.027	0.342
χ		-0.079	0.125	0.529
δ		0.004	0.002	0.023
ε		0.000	0.000	0.258

5.4.2 The effects of dietary treatment on the composition of the abdominal and thoracic contents (organs).

The effects of offering a diet of low protein concentration (L) between day 1 and 5 of lactation and also the effects of offering a diet of high protein concentration over the first 10 days of lactation on the composition of abdominal and thoracic contents (organs) are shown in Table 5.2. Offering diet L between day 1 and 5 ($P<0.01$) reduced the total organ protein content however organ fat and ash contents were not changed. On day 10 of lactation, organs of rats offered diet H contained

($P<0.01$) less fat when compared both to the organs of animals slaughtered on day 1, and to the organs of animals offered diet L and slaughtered on day 5 (Table 5.2). All groups (*i-ix*) , tended to lose organ crude protein between day 5 and 10 of lactation, however only the organs from groups *i-iv* contained less ($P<0.05$) crude protein than organs from animals offered diet L and slaughtered on day 5 (Table 5.5).

Table 5.5 Effects of fixed allowances of crude protein (CP; g/5 d) and non-crude protein gross energy (NCPGE; kJ/5 d) between day 5 and 10 of lactation (groups *i-ix*) on changes in composition of the abdominal and thoracic contents (organs) after a period of protein restriction. Values are mean gains in constituent weights between day 5 and 10 (g)

NCPGE / CP	975	1485	2260	2625	Pooled SED
Organ Protein (g)					
4.55	-1.55^{b}				
8.25	-1.39^{b}				
12.55	-1.59^{b}	-1.41^{b}			0.32
19.1		$-0.54^{b,c}$	$-0.51^{b,c}$		
29.05		-0.92^{b}	$-0.01^{a,c}$	0.46^{a}	
Organ Fat (g)					
4.55	-17.94^{c}				
8.25	$-14.93^{b,c}$				2.47
12.55	$-14.78^{b,c}$	$-13.50^{a,b,c}$			
19.1		-10.86^{b}	-9.34^{a}		
29.05		-16.14^{c}	$-10.74^{a,b}$	-9.17^{a}	
Organ Ash (g)					
4.55	0.14				
8.25	-0.16				
12.55	-0.06	-0.02			0.12
19.1		-0.07	-0.00		
29.05		0.03	-0.03	-0.08	

^{a,b} Means within the same variable with different superscript differ ($P < 0.05$)

The regression analysis suggests that organ crude protein loss was an effect of both dietary NCPGE ($P<0.042$) and CP ($P=0.001$) intake (Table 5.6). Animals from all groups (*i-ix*) slaughtered on day 10 had lost a significant ($P<0.001$) mass of fat

from their thoracic and abdominal contents when compared to the organs of animals slaughtered on day 5, with the largest losses occurring in animals offered the lowest CP and NCPGE rations (Table 5.5). Once again, the regression analysis suggests that limiting NCPGE intake (P=0.006) increases fat mobilisation whereas limiting CP intake has no effect on the mobilisation of organ fat (P=0.281; Table 5.6). The ash content of the organs from animals slaughtered on day 10 (groups *i-ix*) were not significantly different from those on day 5 and they were not influenced by dietary regime.

Table 5.6 Coefficients, standard errors and probabilities for the change in crude protein (CP), and fat composition of abdoninal and thoracic contents (organs) following multivariate regression using the data from rats offered fixed allowances of crude protein (CP) and non-crude protein gross energy (NCPGE), groups *i-ix*, betwen day 5 and 10 after a period of protein restriction. The following model was used: $\hat{Y} = \alpha + \beta gwt + \chi CP + \delta NCPGE + \varepsilon int$. Where int is the interaction term calculated as: int=(CP-mean(CP))*(NCPGE-mean(NCPGE)) and gwt is day 1 maternal gestation weight.

	Adj. r^2 (%)	Estimate	SE	*P*
Organs CP (g)	57.3			<0.001
α		2.39	1.05	0.028
β		0.000	0.003	0.949
χ		0.032	0.015	0.042
δ		0.001	0.000	0.001
ε		0.000	0.000	0.152
Organs Fat (g)	23.0			0.004
α		-11.26	8.90	0.213
β		0.069	0.028	0.018
χ		-0.143	0.131	0.281
δ		0.005	0.002	0.006
ε		0.000	0.000	0.695

5.4.3 *The effects of dietary treatment on the composition of the skin.*

The composition of the skins dissected from rats slaughtered on day 10 and offered diet H (group H) showed no change in total protein content when compared with the skins from animals slaughtered on day 1. However the total fat content ($P<0.001$) decreased from 20.85 (SEM 2.74) g on day 1 to 8.65 (SEM 0.47) g on day 10 (Table 5.2). The skins from rats slaughtered on day 5 after being offered diet L from day 1 of lactation had also lost ($P<0.05$) fat when compared to day 1 values, however this loss was less ($P<0.05$) than that seen in the skins from group H (Table 5.2).

Table 5.7 Effects of fixed allowances of crude protein (CP; g/5 d) and non-crude protein gross energy (NCPGE; kJ/5 d) between day 5 and 10 of lactation (groups *i-ix*) on changes in composition of the skin after a period of protein restriction. Values are mean gains in constituent weights between day 5 and 10 (g)

NCPGE / CP	975	1485	2260	2625	Pooled SED
Skin Protein (g)					
4.55	-1.43^{a,b}				
8.25	-1.88^{b}				
12.55	-0.77^{a,b}	-0.59^{a}			0.48
19.1		-0.77^{a,b}	-1.42^{a,b}		
29.05		-1.61^{b}	-1.01^{a,b}	-0.88^{a,b}	
Skin Fat† (g)					
4.55	-11.44^{b}				
8.25	-9.47^{a,b}				
12.55	-9.93^{a,b}	-8.41^{a,b}			1.76
19.1		-8.80^{a,b}	-7.09^{a,b}		
29.05		-8.75^{a,b}	-7.80^{a,b}	-6.61^{a}	
Skin Ash (g)					
4.55	-0.04				
8.25	-0.04				
12.55	-0.03	0.00			0.10
19.1		-0.04	-0.04		
29.05		-0.01	0.10	0.00	

^{a,b} Means within the same variable with different superscript differ ($P < 0.05$)

† Skin fat composition estimated from the difference between skin DM and total skin protein and ash content.

The regression analysis for skin crude protein clearly indicates that although groups *i-ix* lost protein between day 5 and 10 of lactation it could not be attributed specifically either to NCPGE (P=0.609) or CP (P=0.644) intake; day 1 gestation body weight was the significant parameter (Tables 5.7 and 5.8).

Table 5.8 Coefficients, standard errors and probabilities for the change in crude protein (CP), and fat composition of skins following multivariate regression using the data from rats offered fixed allowances of crude protein (CP) and non-crude protein gross energy (NCPGE), groups *i-ix*, betwen day 5 and 10 after a period of protein restriction. The following model was used: $\hat{Y} = \alpha + \beta gwt + \chi CP + \delta NCPGE + \varepsilon int$. Where int is the interaction term calculated as: int=(CP-mean(CP))*(NCPGE-mean(NCPGE)) and gwt is day 1 maternal gestation weight.

	Adj. r^2 (%)	Estimate	SE	*P*
Skin CP (g)	12.4			0.045
α		5.83	1.63	<0.001
β		0.017	0.005	0.003
χ		-0.011	0.024	0.644
δ		0.000	0.000	0.609
ε		0.000	0.000	0.998
Skin Fat[†] (g)	12.9			0.041
α		-3.44	5.43	0.529
β		0.021	0.017	0.167
χ		-0.014	0.080	0.859
δ		0.002	0.001	0.050
ε		0.000	0.000	0.522

[†] Skin fat composition estimated from the difference between skin DM and total skin protein and ash content.

Fat loss in the skin between day 5 and 10 was greatest in animals from group *i*, offered the least dietary CP and NCPGE, and least in animals from group *ix* which were offered the greatest intake of CP and NCPGE (Table 5.7). The regression analysis attributes this reduction in skin fat loss to an increase in NCPGE intake (P=0.05) and not an increase in CP intake (P=0.859) (Table 5.8). Dietary regimen

between day 5 and 10 had no effect on the ash composition of the skin, and these values are not significant from either those on day 5 or day 1.

5.5 DISCUSSION

The previous trials presented in this thesis have shown that the food intake of lactating rats is sensitive to the protein-energy ratio of the diet. The ability to experimentally manipulate protein and energy intake separately, was therefore constrained by the dietary ratio of protein to energy, as the rats had to eat the entire allocation to fulfill statistical criteria.

Previous studies, using lactating rodents have suggested that well-nourished dams satisfy their increased protein requirement through an increase in food intake and have no need to mobilise protein from an endogenous source (Glore and Layman, 1985; Millican and Vernon, 1987; Pine *et al.*, 1994a,b,d). The tissue analysis of rats offered a high protein concentration diet (diet H) *ad libitum* in the current study support this principle, although it should be noted that there was no intermediate slaughter group between day 1 and 10, so any protein mobilised during the first few days may well have been replenished by day 10.

In a previous serial slaughter trial (Pine *et al.*, 1994a), the carcass composition of females slaughtered at the end of the trial was estimated for earlier slaughter points by linear regression *v.* body weight and composition of females offered similar dietary treatments and slaughtered earlier in lactation. When the same analysis was applied to the data from this experiment, predicted values for carcass protein and fat were unexpectedly low (11 % and 27 % respectively) for animals which had consumed the low protein to energy ratio diets; groups *i* (n=3) and *ii* (n=3) when compared to similarly treated animals in the previous work (Pine *et al.*, 1994a). Some animals from these two groups only, had been excluded (n=3 and n=4 respectively) from the data analysis as they had not eaten their complete ration over the 5 day period. Analysis of the data revealed that animals which had consumed their ration, and were therefore included in the experimental analysis tended to be lighter on day 5 of

lactation than those that had not eaten their ration (321 (SEM 15.0) g as opposed to 346 (SEM 6.8) g (P=0.141)). Further analysis of the data showed that the weight gain between day 1 of gestation and day 5 of lactation of animals that had eaten their entire allocation of diet with a low protein concentration between day 5 and 10 of lactation was less than that of animals which had not eaten their ration (3.6 (SEM 4.9) g as opposed to 33.8 (SEM 6.3) g (P=0.004)). As it was not possible to predict whether or not the animals slaughtered on day 5 (and used to develop the regression equations) would have eaten a low protein concentration diet between day 5 and 10, linear regression analysis was not used to predict the day 5 tissue composition of animals slaughtered on day 10. Instead, it was assumed that the mean composition for animals slaughtered on day 5 was a representative estimate and so protein and fat gains between day 5 and 10 were estimated by subtraction of the day 10 value from the mean day 5 value. This finding is of great interest as it lends support to the metabolic embarrassment theory of food suppression proposed by Pine *et al* (1994a) and Jessop (1996a). Animals which consumed protein-energy imbalanced diets between day 5 and 10, were those which had both increased their fat reserves the least during gestation and lost the most body weight between day 1 and 5 of lactation. These animals would be expected to have much less body fat to mobilise between day 5 and 10 and therefore were much more likely to consume a high energy, low protein diet, if food intake was being at least partly suppressed through a situation of metabolic embarrassment. Indeed, analysis of carcass, organ and skin fat after slaughter of these two groups, showed that the animals which consumed the low protein concentration diets between day 5 and 10 contained less fat (P=0.03) than those which did not. These animals were all of the same strain, age, parity (one previous litter), were kept under the same environmental conditions and were offered the same high protein concentration diet throughout gestation. The only obvious biological reason for some animals to store more energy yielding nutrients than others is differing demands of the foetal and placental tissue. Although it is not easy to determine the mass of the placenta, as the mother consumes it soon after birth, analysis of the litter masses at birth showed no significant difference between these two groups of animals. As fat accumulation during gestation is under the control of

progesterone (Hervey and Hervey, 1967) differences in total adipose tissue may be due to differing plasma progesterone concentration or receptor sensitivity as a result of inter animal variation. Animal variation in fat deposition during gestation has previously been noted in rodents (Spray, 1950; Beaton *et al.*, 1954) though these authors offered no explanation as to the reason why. The concept of carcass fat content at the time of parturition affecting subsequent nutrition has been well documented for dairy cows (Bines *et al.*, 1969; Swick and Benevenga, 1977; Garnsworthy and Topps, 1982; Garnsworthy and Jones, 1987). In addition, the study of Garnsworthy and Jones demonstrated that offering a low protein diet during lactation has an increased suppression on food intake on fat cows when compared to all others (Garnsworthy and Jones 1987), in agreement with this work.

Offering an unbalanced diet, (low protein - high energy), diet L, immediately following parturition until day 5, led to no significant loss of body protein when compared with animals slaughtered on day 1. This was surprising as work in growing rats offered a protein free diet, suggested that crude protein losses from both the carcass and skin declined rapidly over the first few days (Allison and Wannemacher, 1965). However, these rats were offered a protein free diet, which is an extreme situation and the rats were also immature (200-250 g) and would not, therefore, possess sufficient fat stores and so body protein may have been catabolised as a source of energy. The work of (Pine *et al.*, 1994d) using rats maintained under similar conditions to those in the present study and offered diet L post-partum, suggested that the rate of skeletal muscle (gastrocnemious) protein loss was greatest between day 1 and 8 of lactation where it lost 31 % of its original protein content. But, protein loss of this muscle over the first 4 days was not significant, declining from (0.385 (SEM 0.022) g) on day one of lactation to (0.317 (SEM 0.025) g). This work only analysed the gastrocnemius muscle, liver and mammary gland and therefore data for total carcass crude protein are not known. It is quite possible that mass protein mobilisation of the carcass is spared until labile reserves of specific muscle groups, of which the gastrocnemius may be one, have been depleted. This situation would agree with the work of Cherel *et al.* (1991) who showed that mature, non-lactating rats which underwent a 9 day period of starvation spared both carcass and

skin crude protein until day 7. It is quite likely that crude protein determination by the Kjehdahl method is not sensitive enough to pick up the very small protein losses seen in the gastrocnemius, and perhaps the other muscle groups. Between day 5 and 10, the protein content of the carcasses and skins from animals offered low CP - low NCPGE diets decreased significantly and so by day 10, the losses were comparable to the work of (Pine *et al.*, 1994a). This loss of carcass protein was reduced as diet quality improved, and the regression analysis (Table 5.4) showed the dietary CP intake to be the significant component (P=0.048). The regression coefficient for crude protein intake, although significant was remarkably small: a 1 g increase in crude protein intake would only spare 0.17 g of body protein. Efficiency of use of the first limiting amino acid may be assumed to be 85 % (Oldham, 1987) and therefore the 17 % efficiency observed here would be expected to be higher. The mammary gland procures a high priority for nutrients during lactation (Bauman and Currie, 1980). It is quite plausible that a greater proportion of the extra dietary protein is being partitioned towards the mammary gland for production of milk and not towards skeletal muscle. Increasing dietary protein intake may halt the increase in the muscle fractional degradation rate and also relieve the suppression in the fractional protein synthesis rate (Pine *et al.*, 1994d).

Although skin protein content was significantly reduced over this period, particularly in animals receiving the low CP - low NCPGE regimes, the regression analysis did not attribute it to either CP or NCPGE intake (Table 5.8), body weight at the time of conception was the significant coefficient. A period of 9 days starvation has been reported to reduce skin protein content of mature, non-lactating rats by values comparable with this study (Cherel *et al.*, 1991). The decline in the skin protein content has been reported to be due to a decrease in the fractional protein synthesis rate of this tissue (Cherel *et al.*, 1991) which may be due to a decline in skin blood flow, limiting substrate availability (Harris *et al.*, 1994). If this were the case, protein would be spared for utilisation by other organs, for instance the mammary gland, through the reduced protein turnover in the skin. This may well explain the lack of hair growth noted in animals offered a low protein concentration diet in this

and the previous work, and also in humans suffering from protein - energy malnutrition (Alleyne *et al.*, 1977).

The total protein content of the abdominal and thoracic contents fell between day 1 and 5 of lactation, presumably as a result of organ hypotrophy. The organs of animals offered the low CP and NCPGE diets continued to lose a significant mass of protein between day 5 and 10, however, protein loss was halted for rats offered more balanced diets, in terms of protein and energy. Organ hypotrophy has been shown to occur after prolonged food restriction in the rat (Sakanshi *et al.*, 1987; Pine *et al.*, 1994a), and in growing lambs (Wilkinson and Stark, 1987). A reduced food intake, as a result of offering low protein : energy ratio diets would not require such an extensive gastrointestinal tract and therefore hypotrophy would be a source of both protein and fat for milk production. Food intake increased markedly when more balanced diets were offered and the catabolised protein would need replacing. The regression analysis (Table 5.6) shows this response to be associated with both CP and NCPGE intakes (P=0.042 and P=0.001, respectively). This is not surprising as it is generally accepted that the organs of the gastrointestinal tract have a high protein turnover rate which is expensive in terms of energy as well as protein. The results for organ protein content presented in this study should however be treated with a degree of caution as the stomach and intestine were left intact and so their contents would have been included in the analysis, diet protein content may therefore have effected the results.

The fat content of the skins, including hair was estimated as the difference between the dry mass and the protein and ash masses. The skins could not be processed for calorimative evaluation. To determine the accuracy of this method, the carcass and organ fat contents were determined by subtraction and compared with those determined by analysis; the difference was less than 2 %. Both carcass and skin fat contents decreased significantly during this 4 day protein restriction period, but organ fat did not. Between day 5 and 10 carcass, organ and skin fat (P<0.01) declined in all groups (*i-ix*), with losses shown for animals offered low NCPGE regimes tending to be greater, though not significantly so, Tables 5.4, 5.6 and 5.8.

Naismith *et al.* (1982) concluded that as fat mobilisation during lactation in rats was not related to lactational performance it was under hormonal and not dietary control. However, the regression analysis from the present study showed fat mobilisation in the carcass, organs and skin, to be significantly related to NCPGE intake (Tables 5.4, 5.6 and 5.8) and therefore must also be related to food intake. The regression analysis for carcass, organ and skin fat shows that although the coefficient for CP intake is not significant, it is always negative (Tables 5.4, 5.6 and 5.8). This would suggest that as CP intake increases body fat tends to decrease; indicating that dietary protein is required for the rate of endogenous adipose catabolism to be maximum.

Bone mobilisation can be indirectly stimulated when dietary anion-cation balance is reduced (Block, 1988). Mineral contents as measured by ash content did not significantly alter with time or dietary treatment group in this study. This is not surprising as all diets were designed to provide an adequate mineral intake to support lactation (National research Council, 1978).

In summary it may be concluded that when lactating rats are subject to a period of severe dietary protein restriction, carcass and skin protein is spared for the first 5 days. After this time, offering diets of low crude protein resulted in significant mobilisation of carcass, organ and skin protein. Mobilisation of body fat occurs, irrespective of dietary treatment, however offering low NCPGE diets after a period of protein restriction tended to increase these losses.

CHAPTER 6

TRIAL 4

Sensitivity of Mammary Secretory Cell Turnover to Protein Restriction and Re-alimentation

6.1 ABSTRACT

This study investigated the influence of protein undernutrition and re-alimentation on secretory cell proliferation and death in the mammary gland of rats. During gestation, female Sprague-Dawley rats were offered a diet with a high protein concentration (H; 215 g crude protein (N×6.25; CP)/kg dry matter (DM)); litters were standardised to 12 pups at parturition. During lactation, rats were offered diet H, or a low protein diet (L; 90 g CP /kg DM) for the first 7 days of lactation (treatments HH and LL) or diet L transferring to diet H on day 6 of lactation (LH). On day 7 of lactation, the incorporation of [^{3}H]-thymidine into mammary explants using media of differing composition (Dulbecco's Phosphate Buffered Saline (D-PBS); Medium 199 (M199); Medium 199 + Bovine Foetal Serum (20 % v/v)(FBS)) was studied over 4 h *in vitro*. Tissue was prepared for histological and autoradiographic examination. In addition, low molecular weight DNA was extracted from mammary tissue from rats used in a previous experiment in order to assess cell death.

[^{3}H]-Thymidine was incorporated at a much faster rate in group LH when compared to group HH when incubated in either M199 or FBS. Tissue from group LH incubated in D-PBS did not show this increase in incorporation rate, the rate being similar to tissue incubated from group HH. The histological study showed that the epithelial cells of rats offered diet L had significantly more cells undergoing pyknosis on day 7 of lactation when compared to rats offered diet H. Transferring to diet H after 6 days protein restriction reduced this epithelial cell death rate to a level comparable with rats offered diet H from day 1 of lactation. All dietary treatments resulted in a high mass of low molecular weight DNA extracted from mammary tissue, although tissue from rats offered diet L tended to contain more. There were no signs of apoptosis.

These results suggest that the improved lactational performance of rats re-alimented with diet H after a 6 day period of severe protein restriction is a consequence of a high rate of epithelial cell proliferation and reduced rate of cell death

6.2 INTRODUCTION

Mammary cell mass has been shown to increase post-parturition in the well-nourished mouse (Brookreson and Turner, 1959; Traurig, 1967; Knight and Peaker, 1982, Shipman *et al.*, 1987), goat (Knight and Peaker, 1984b) and rat (DeSantiago *et al.*, 1991 and Chapters 2 and 4). This development did not occur if a diet of low protein to energy ratio was offered, although, the gland retained the ability to increase total cell mass if the dietary protein to energy ratio of the diet was increased up to mid lactation (Chapter 2). The suppression of mammary development was shown to be a consequence of the restricted energy intake resulting from the reduced food intake associated with offering lactating rodents diets with a low protein high energy ratio (Chapter 4).

Since both secretory cell number and activity are components of milk yield, the factors controlling cell turnover are of crucial importance. Knight and Peaker (1982a) suggested an exponential increase in the epithelial cell population of the mammary gland between mid-pregnancy and day 5 of lactation for well nourished mice, after which the cell population remained stable before declining. There are no published data on rates of cell death in the mammary gland nor on nutritional influences on cell proliferation and cell death for mammary tissue.

The primary objective of this experiment was to study mammary epithelial cell turnover during early lactation.

6.3 MATERIALS AND METHODS

6.3.1 Experimental Protocol

Thirty one multiparous (second parity) female Sprague-Dawley rats (B & K Universal Ltd., Hull) weighing on average (302 (SE 7.2) g) were housed in a room regulated at 22 °C with a relative humidity between 45-65 % and with a light period from 07:00-19:00 hours for a minimum of two weeks before breeding. Females were then placed in a wire bottomed cage with a proven male breeder. Day 1 of gestation was the morning on which mating was confirmed through the presence of a vaginal plug, after which the females were caged individually in solid-bottomed plastic cages for the remainder of the experiment.

From day 1 of gestation the females were offered *ad libitum*, a diet with a high protein concentration (H; 215 g crude protein (N×6.25; CP) / kg dry matter (DM)), formulated to meet NRC (1978) requirements for vitamins and minerals until parturition, which was designated day 1 of lactation. On this morning, litters were standardised to 12 pups to ensure a uniform and high lactational demand. Litters that could not be standardised to 12 by cross fostering with pups born on the same day were removed from the trial, along with their mothers.

During lactation two isoenergetic diets were offered *ad libitum* one of high protein concentration, diet H, and one of low (L; 90 g CP /kg DM) protein concentration. The protein source for both diets was casein supplemented with DL-methionine (99:1 w/w) (Table 6.1). The diets were formulated to provide 21 MJ gross energy (GE) kg^{-1} DM with a constant carbohydrate energy : fat energy ratio of 2.3:1. Lactational dietary treatments were *ad libitum* supply of either diet H (HH; n=10) or diet L (LL; n=7) for the first 7 days of lactation or diet L with transfer to diet H (LH; n=7) on day 6 of lactation. This dietary allocation produced three groups of females (LL, HH and LH) the first letter representing the dietary treatment from day 1 of lactation to day 6 and the second from day 6 to 7.

Dam body weights and food intakes were recorded at the same time each day throughout the experiment as were standardised litter weights. All females were given free access to fresh drinking water.

All dams were killed on day 7 of lactation when a snippet of the left inguinal mammary gland was removed from each animal.

Table 6.1 Composition of diets H and L (g/kg DM)

	High (H)	Low (L)
Casein : methionine (99:1 w/w)	215	90
Maize oil	192	230
Corn starch : sucrose (2:1 w/w)	444	530
Vitamin mix	50	50
Mineral mix	50	50
Maize flour	40	40
Choline chloride	7	7
Antioxidant[‡]	0.013	0.013
Emulsifier[§]	2	2

[†] Mineral and vitamin mixtures were formulated to meet NRC (1978) requirements.
[‡] Antioxidant - butylated hydroxy toluene (g/kg fresh weight).
[§] Emulsifier - egg lecithin (g/kg fresh weight).
All diets were isoenergetic with constant carbohydrate energy to fat energy ratio (2.3:1)
Diet Analysis: Protein (g CP/kg DM) H 215 (SEM 0.32); L 91 (SEM 0.06)
Gross energy (MJ/kg DM) H 21.2 (SEM 0.26); L 21.4 (SEM 0.14)

6.3.2 [^{3}H]-Thymidine incorporation - liquid scintillation

Incorporation of [^{3}H] thymidine was quantified by liquid scintillation after developing a suitable method from Knight and Peaker (1982a) and Woodward *et al.* (1993). The final protocol was as follows. A snippet of mammary tissue was removed from the left inguinal mammary gland of each rat and chopped, under a small amount of the medium they were to be incubated in, into slices with a width of 500 μm using a tissue chopper. The slices were washed in more medium (37 °C) to

remove the cell contents of cells disrupted during the chopping procedure. Two slices (0.005-0.010 g) per medium and per incubation time were accurately weighed (4 d.p.) and placed directly into separate Bijou jars, each containing 2 cm^3 of pre-warmed (37 °C) medium. All media were obtained from Sigma Chemicals, Poole, U.K. The media used were Dulbecco's phosphate buffered saline (D-PBS); Medium 199 (M199) and Medium 199 + 20 % (v/v) Foetal bovine serum (FBS); each 2 cm^3 aliquot of media contained 1.84 MBq [methyl-^{3}H] thymidine (Amersham International plc, Buckinghamshire, UK). Samples were incubated in each of the three media, in duplicate in a shaking water bath for a period of 1, 2 or 4 hours. At the end of the incubation period the medium was quickly removed by aspiration and the samples washed three times in 2 cm^3 medium at 4 °C, each wash being carefully removed by aspiration. The tissue was then homogenised in 2 cm^3 TCA (10 % v/v) using a Potter-Elvehjem, glass / teflon homogeniser, transferred to a 2 cm^3 plastic, capped centrifuge tube and left to stand for 10 min at 4 °C, before being centrifuged for 5 min at 1000×g. The supernatant was discarded, and the pellet washed in 1 cm^3 TE buffer; centrifuged for 5 min at 1000×g and the supernatant discarded. The entire tube was added to a scintillation vial (Beckman, micro-vial) and 4 cm^3 scintillation cocktail added. This procedure had previously been found to remove all unincorporated [^{3}H] thymidine. Incorporated [^{3}H] was determined by liquid scintillation spectrometry (Beckman LS 5000CE), and the results expressed as d.p.m μg^{-1} DNA.

DNA was quantified from a known (4 d.p.) mass (0.004 - 0.008 g) of a similar snippet of mammary tissue removed at the same time and homogenised in 2 cm^3 of TE buffer using a Potter-Elvehjem, glass / teflon homogeniser. DNA concentration was determined using PicoGreen Nucleic Acid Quantitation Reagent (Molecular Probes Europe BV, The Netherlands).

In order to ensure that only thymidine incorporation was studied as opposed to thymidine uptake, the above protocol took time and rats to develop. As a result, the initial dietary regimen had to be reduced to two groups, HH and LH in order to obtain satisfactory treatment group sizes.

6.3.3 Low Molecular Weight DNA Extraction and Labelling

Low molecular weight DNA was extracted from mammary samples of rats kept under similar conditions in a previous experiment (trial 1) and these results are presented here. Briefly, the rats from trial 1 were offered diet H throughout gestation, and at parturition litters were standardised to 12 pups and the dams were offered *ad libitum* one of two diets H, or L at the same formulation as H or L used in this trial. Either, diet H (HHH) or diet L (LLL) was supplied for the first 12 days of lactation, or diet L transferring to diet H on either day 6 (LHH) or day 9 (LLH) of lactation. On day 1, 6 (L, H), 9 (LL, LH) and 12 (LLL, LLH, LHH, HHH) of lactation rats from each group ($n \geq 6$) were slaughtered by decapitation and mammary tissue was removed immediately post mortem in order for the low molecular weight DNA to be extracted.

The DNA was extracted by the method of Tilly and Hseuh (1993). This method ensures all the low molecular weight in addition to the high molecular weight DNA is extracted.

The DNA was visualised using ethidium bromide or 3' end labelled with [α-^{32}P] ddATP using the method of Tilly and Hseuh (1993) and run on agarose (1.5 % w/v) gels electrophoretically.

6.3.4 Histology and Autoradiography

Two chopped pieces (5-10 mg) of mammary tissue per rat were incubated at 37 °C in M199 containing 185 Bq [methyl-3 ^{3}H] thymidine for 1 hour. The medium was removed by aspiration and the tissue washed three times in 2 cm^3 of unlabelled media before being fixed for 24 h in Bouin's fixative (formaldehyde, picric acid and acetic acid). After the fixative was removed by aspiration, the tissue was dehydrated by progressively washing in increasing concentrations of ethanol (70, 90, 95, and 100 % for at least 1 h each). Tissue was subsequently placed in cedar wood oil for 24 h. The cedar wood oil was then replaced with toluene. After 30 min, the toluene was removed, and the tissue given 4 changes of warm paraffin wax. Finally the tissue was

blocked out in paraffin wax using an appropriate mould. Sectioning began after the blocks were fully hardened (about 24 h). The sections were cut at a thickness of 5 μm with a microtome. Sections were floated on water in a heated waterbath (50 °C), mounted on gelatin covered slides and allowed to dry (20 °C) overnight before staining. Slides were dewaxed in toluene for 15 minutes and stained with Harris' Haematoxylin for 5 min. Slides were dipped in emulsion gel (K.5, Ilford Scientific Products), developed (Kodak D-19) and fixed.

Individual slides were examined (×400) to determine the labelling index (proportion of cells with autoradiographic grains over the nucleus) and also an index as to the number of cells undergoing necrosis. Unfortunately, the uptake of [methyl-3 ^{3}H] thymidine was restricted to only a few slides and so no labelling index was determined by this method. In order to obtain a representative sample, the necrotic index for each dietary group (LL, *n*5; LH, *n*5; HH, *n*5) was determined by counting the number of cells showing uniformly compacted chromatin (pyknosis) in 10 alveoli units, every fifth section for the whole explant. Representative photographs were also taken.

6.3.5 Statistical Analysis

The data for dam food dry matter intake, body-weight change and standardised litter growth rate were cumulated (day 1-6 and day 7) and analysed by two way analysis of variance, with dam day 1 gestation body-weight as a covariate (Genstat5 ver. 3.1). Least significant differences were calculated and *t* tests were performed to compare sample means between dietary treatment groups.

The liquid scintillation spectrometry data were analysed using two way analysis of variance blocking for rat (Genstat5 ver. 3.1). Least significant differences were calculated and *t* tests were performed to compare sample means between dietary treatment groups, culture media, and incubation time. Linear regression analysis was performed on the data to determine rates of [^{3}H] thymidine incorporation for each of

the treatment groups and these rates were compared using the table of contrasts derived from the analysis of variance.

The necrotic index data were analysed using two way analysis of variance (Genstat5 ver. 3.1). Least significant differences were calculated and *t* tests were performed to compare sample means between dietary treatment groups.

6.4 RESULTS

6.4.1 Maternal body-weight changes, food intakes and litter-weight gains.

The results for groups LL, LH and HH are summarised in Figures 6.1 and 6.2 and Table 6.2. By day 6, group LL had lost body weight ($P<0.001$) and were lighter ($P<0.001$) than group H which had effectively maintained a constant weight.

Dams previously offered Diet L for the first 6 days of lactation, showed a rapid ($P<0.05$) increase in body weight when transferred to diet H, but did not regain the weight lost over the first 6 days (Figure 6.1 and Table 6.2).

Table 6.2 Maternal body weight change, feed intake and standardised litter weight gain of rats offered either solely diet L (LL) or solely diet H (HH) for the first 6 days of lactation or diet L with transfer to diet H after 6 (LH) days of lactation. Means with different superscripts within the same row are significantly different ($P<0.05$).

	Lactation diet			
	LL	**LH**	**HH**	**Pooled SED**
Dam weight change (g)				
Day 1 - 7	-64.2[a]	-33.9[b]	-8.5[c]	9.17
Day 1 - 6	-59.0[a]	-48.5[a]	-8.3[b]	6.07
Day 6 - 7	-5.2[b]	14.6[a]	-0.2[b]	6.63
Dam feed intakes (g)				
Day 1 - 7	64.6[b]	77.4[b]	156.4[a]	16.13
Day 1 - 6	60.2[b]	50.4[b]	121.8[a]	13.46
Day 6 - 7	4.4[c]	27.0[b]	34.6[a]	3.40
Litter weight gains (g)				
Day 1 - 7	42.0[b]	53.6[b]	108.4[a]	9.20
Day 1 - 6	38.7[b]	39.1[b]	84.7[a]	7.66
Day 6 - 7	3.3[c]	14.6[b]	23.7[a]	2.23

Food dry matter intakes of Diet L initially rose, but not as rapidly as the intakes of rats offered Diet H and after day 4, the intakes of the two groups diverged. Consequently, this resulted in a much greater total food intake (g DM) for females offered Diet H compared to those offered Diet L ($P<0.001$; Table 6.2 and Figure 6.1). Group LH showed a highly significant ($P<0.001$) increase in food intake when offered Diet H after 6 days protein restriction when compared to group LL.

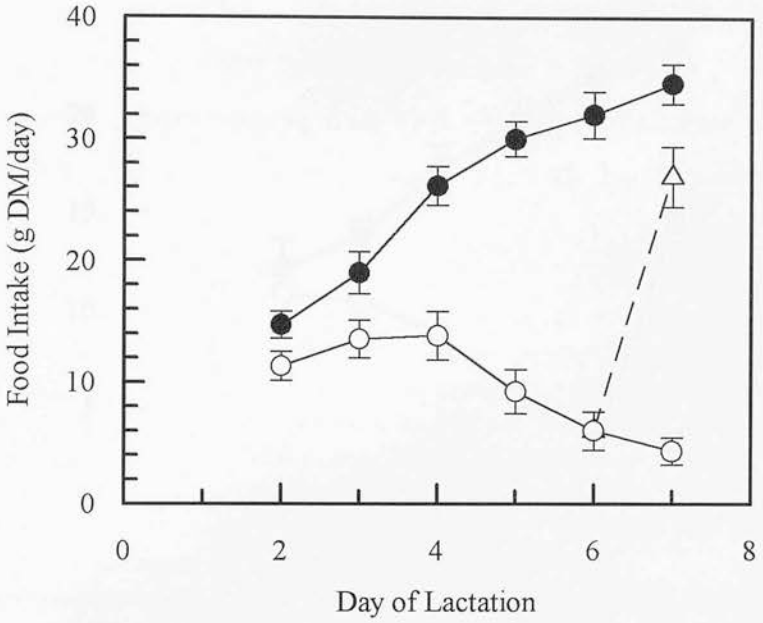

Figure 6.1 Daily food dry matter intake (g) of rats offered either a low (L)- or high (H)- protein diet for the first 7 days of lactation, or diet L with transfer to diet H after 6 days of lactation: (–○–), LL (*n5*); (–△-), LH (*n8*); (–●–), HH (*n8*). Values represent a mean and SEM, using dam day 1 gestation weight as a covariate.

Lactational performance was estimated by the weight gain of a standardised litter and closely reflected food dry matter intake (Figures 6.1 and 6.2). The greater supply of both dietary energy and protein allowed litters in group HH to achieve a greater weight gain during lactation when compared to those offered Diet L. Changing from Diet L to H after day 6 allowed group LH to increase lactational performance with significant improvements in litter weight gain being seen within 24 hours of the dietary change.

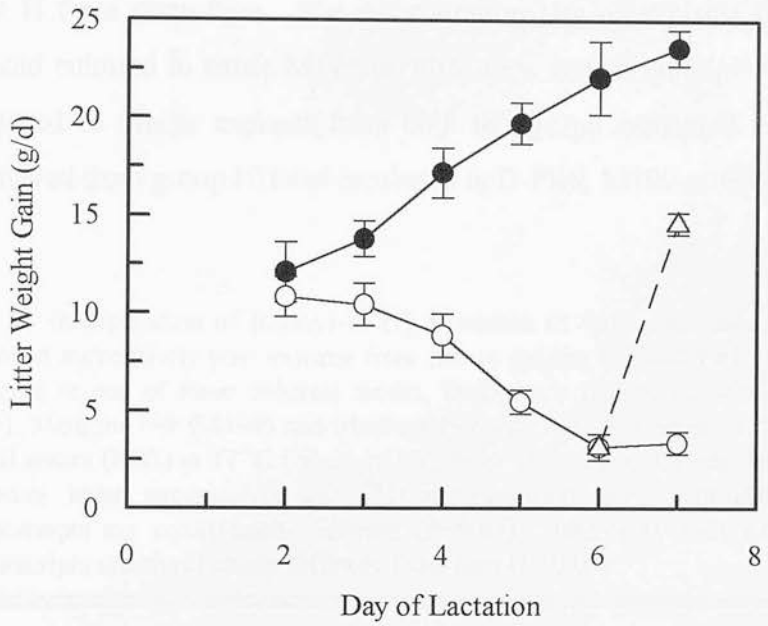

Figure 6.2 Daily litter weight gain (g) of rats offered either a low (L)- or high (H)-protein diet for the first 7 days of lactation, or diet L with transfer to diet H after 6 days of lactation: (–○–), LL (*n5*); (–△-), LH (*n8*); (–●–), HH (*n8*). Values represent a mean and SEM, using dam day 1 gestation weight as a covariate.

6.4.2 Epithelial Cell Proliferation

Both the total quantity and rate of [^{3}H] thymidine incorporation in mammary explants cultured *in vitro* for either 1, 2 or 4 h are summarised in Figure 6.3 and Table 6.3. As the method involved significant development to ensure the results were an accurate measure of thymidine incorporation it was only possible to carry out the final procedure using groups LH (n=3) and HH (n=3).

Choice of media had no effect on the rate of [^{3}H] incorporation in explants removed from rats offered diet H from day 1 of lactation. These samples were, however, still incorporating thymidine and total incorporation in the samples incubated for 4 h were higher (P<0.01) than those incubated for 1 h (Table 6.3).

Explants removed from rats in group LH which were cultured in D-PBS did not show significantly different rates of [^{3}H] incorporation when compared to rats

offered diet H from parturition. The incorporation rate of explants removed from group LH and cultured in either M199 or FBS were considerably ($P<0.001$) higher when compared to similar explants from both this group incubated in D-PBS and samples removed from group HH and incubated in D-PBS, M199 or FBS (Table 6.3).

Table 6.3 Incorporation of [methyl-3 ^{3}H] thymidine *in vitro*, into mammary explants removed immediately post mortem from rats in groups LH and HH. Explants were cultured in one of three different media, Dulbecco's phosphate buffered saline (D-PBS), Medium 199 (M199) and Medium 199 with the addition of 20 % (v/v) bovine foetal serum (FBS) at 37 °C for either 1 h, 2h or 4h (see text for details). Means with different letter superscripts and [^{3}H] incorporation rates with different symbol superscripts are significantly different ($P<0.05$). Intercepts with different symbol superscripts are significantly different from zero ($P<0.05$).

		Lactation Treatment						
		LH			HH			
Media		**D-PBS**	**M199**	**FBS**	**D-PBS**	**M199**	**FBS**	**Pooled SED**
Total incorporation								
Time (h)	**1**	1198^{a}	$3315^{a,c}$	1384^{a}	1855^{a}	1722^{a}	1933^{a}	
	2	$2146^{a,b}$	11842^{e}	11358^{e}	3353^{a}	$4033^{a,d}$	$4387^{a,d}$	1636
	4	$4155^{a,b}$	27988^{f}	27414^{f}	$5467^{b,c,d}$	6794^{d}	$6587^{c,d}$	
Regression Analysis								
Adj. r^2 (%)		64.7	93.7	96.1	56.2	50.7	67.6	
P		0.006	0.047	<0.001	0.012	0.019	0.004	
Intercept		193.3	-4757.0^{*}	-6644.0^{*}	798.1	342.0	832.7	
Rate‡		988.5^{*}	$8202.4^{\S}$	$8584.0^{\S}$	1183.0^{*}	1646.1^{*}	1487.0^{*}	

† Total [^{3}H] thymidine incorporation (d.p.m./μg DNA)
‡ Rate of [^{3}H] thymidine incorporation (d.p.m./μg DNA/h)

There was no significant difference in the rates of [^{3}H] incorporation between samples cultured in either M199 or FBS from group LH and total thymidine incorporation was significantly ($P<0.001$) higher at both the 2 and 4 h sampling points, but not after 1 h.

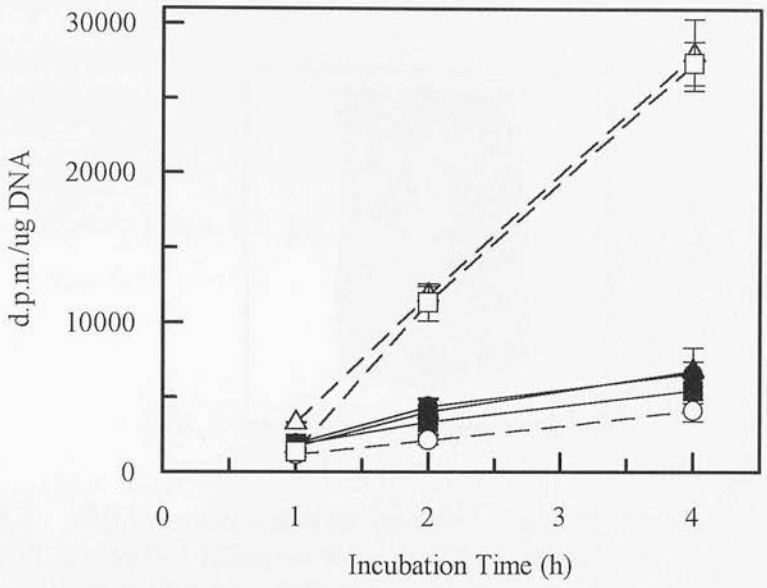

Figure 6.3 Total incorporation of [methyl-3 ^{3}H] thymidine *in vitro*, into mammary explants removed immediately post mortem from rats in groups LH and HH. Explants were cultured in one of three different media, Dulbecco's phosphate buffered saline (D-PBS), Media 199 (M199) and Media 199 with the addition of 20 % (v/v) bovine foetal serum (FBS) at 37 °C for either 1 h, 2h or 4h. This led to 6 groups (-○-), LH[D-PBS](n=3); (-△-), LH[M199](n=3); (-□-), LH[FBS](n=3); (-●-), HH[D-PBS](n=3); (-▲-), HH[M199](n=3); (-■-), HH[FBS] (n=3) (see text for details).

6.4.3 Epithelial Cell Death

The results of the molecular weight extraction from animals from trial 1 are shown in Figures 6.4 and 6.5. As the DNA labels used in Figures 6.4 and 6.5 are different, quantitative comparisons cannot be made. However, Figure 6.4 clearly shows that the mammary tissue of rats offered diet L for the entire 12 day period studied contained a considerable mass of low molecular weight DNA, although the ladder pattern characteristic of cells undergoing apoptosis is not evident. There is a considerable quantity of low molecular weight DNA present in all of the other dietary treatments as shown in Figure 6.5, although more is evident in the tissue extracted from rats in group L. Once again there is no evidence of DNA having arisen from cells undergoing apoptosis.

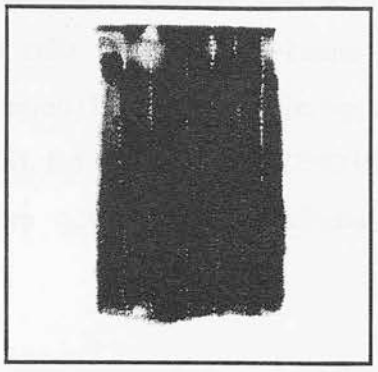

Figure 6.4 DNA extracted from the mammary glands of 7 rats used in trial 1 and offered diet L for the first 12 days of lactation (LLL); labelled with ^{32}P and run on an agar gel for 90 min at 60 amps. Each lane of the gel is the DNA sample from each rat. See Chapter 2 for a full description of the experimental design.

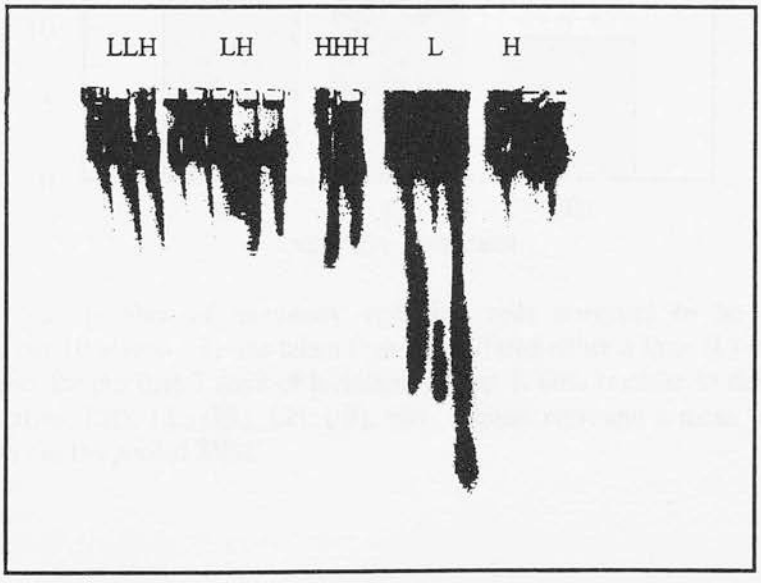

Figure 6.5 DNA extracted from the mammary glands of rats used in trial 1 and offered diet H or L; labelled with ethidium bromide and run on an agar gel for 3 h at 60 amps. Each lane of the gel is the DNA sample from one rat. See Chapter 2 for a full description of the experimental protocol

The effects of lactational dietary treatment on the number of cells undergoing pyknosis are summarised in Figure 6.6. Offering diet L during early lactation led to significantly ($P<0.001$) more cells undergoing pyknosis when compared to rats offered diet H over the same period (Figure 6.6). Offering diet H after a 6 day period of protein restriction, group LH, led to a reduction ($P<0.001$) in the number of cells undergoing pyknosis to a value not significantly different from group HH (Figure 6.6).

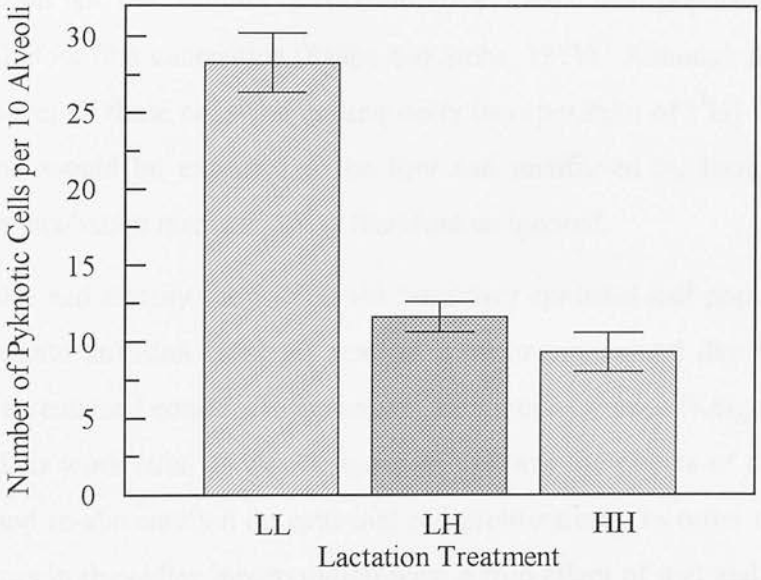

Figure 6.6 Mean number of mammary epithelial cells observed to be undergoing pyknosis per 10 alveoli. Tissue taken from rats offered either a low- (L) or high (H)-protein diet for the first 7 days of lactation, or diet L with transfer to diet H after 6 days lactation: (▨), LL; (▩), LH; (▧), HH. Values represent a mean (n=80), and error bars are the pooled SEM.

6.5 DISCUSSION

The primary objective of this study was to determine whether the increase in cell mass after re-alimentation with a diet of high protein to energy ratio following a period of protein restriction, seen in Chapters 2 and 4, arose through either an increase in the rate of cellular proliferation, a decrease in cell death or a combination

of both. The results show that both cell proliferation and death rate are extremely sensitive to nutrition; this has not previously been reported.

Voluntary food intake of a diet with a low protein to energy ratio (diet L) was considerably reduced in rats compared to rats offered a higher ratio diet. This resulted in an impaired lactational performance, as indicated by the daily litter weight gain of a standardised litter, in agreement with the three previous trials (Chapters 2, 3 and 4).

The rate of cellular proliferation may be assumed to be due to the epithelial cell population as the adipose and connective tissue cell population is largely determined before first conception (Paape and Sinha, 1971). Although there may be a natural turnover of these cells and consequently incorporation of [^{3}H] thymidine, the incorporation would be expected to be low and unaffected by lactational dietary treatment or incubation media and may therefore be ignored.

Trial 1 had already shown that the mammary epithelial cell population of rats offered adequate nutrition (Diet H) reached a maximum around day 6 of lactation after which it remained constant in agreement with work for mice (Knight and Peaker, 1982b,c). This work (trial 4) was designed to examine the effects of dietary protein restriction and re-alimentation on epithelial cell proliferation. In order to ensure that the differences in thymidine incorporation were a true effect of diet and not of media or other artifact, explants were incubated in three different media. Mammary explants were chosen in preference to more refined culture models because the time delay between slaughter and incubation was reduced, increasing the likelihood of obtaining results similar to the *in vivo* situation. Given that it is now clear that regulatory signals are provided by both the mammary epithelial cells themselves (Ip and Darcy, 1996) and also the cells of the stromal tissue (Imagawa *et al.*, 1994) maintaining the tissue's integrity is important if the *in vivo* situation is to be modelled successfully.

Dulbeco's phosphate buffered saline (D-PBS) is a balanced mixture of inorganic ions in order to maintain the pH and osmotic balance of the medium; it contained no protein or energy yielding nutrients and rates of incorporation would therefore be expected to be a true indication of the dietary treatment. However,

mammary tissue taken from lactating glands is highly active and there would be a considerable nutrient demand over the 4 h incubation period. Tissue incubated in D-PBS may be either protein and/or energy limited; this possible nutrient limitation may inhibit mitosis. Therefore tissue was also incubated in Medium 199, which is generally considered complete in terms of inorganic salts, energy, amino acids and vitamins. In addition to nutrients, hormones and paracrine regulatory factors, a number of growth factors, for example epidermal growth factor (EGF) (Tonelli and Sorof, 1980; Imagawa *et al.*, 1985) and transforming growth factor-α (TGF-α) (Ip *et al.*, 1992) are known to be required for mammary epithelial cells to undergo mitosis. It is widely accepted that foetal bovine serum contains, amongst other things, an ill-defined quantity of growth factors. FBS was therfore included at 20 % (v/v) in M199 as a positive control to ensure the epithelial cells would proliferate, if predisposed to do so.

Explants removed from rats offered diet H for the first 7 days of lactation showed a low but steady rate of proliferation, as determined by *in vitro* incorporation of [^{3}H]-thymidine in agreement with work in mice (Knight and Peaker, 1982b). As total epithelial cell mass may be assumed to be stable at this time, the rate of incorporation (Table 6.3) represents natural cell turnover of these cells. The rate of cellular proliferation of tissue from these rats was not affected by the incubation media. This would suggest that mammary epithelial cells removed from well nourished rats and cultured within their normal stromal environment contain sufficient nutrients and other factors required for mitosis to occur at a relatively low rate for at least a 4 h incubation period. This was in contrast to tissue removed from rats on day 7 of lactation which had been offered diet H for the previous 24 h after 6 days of being offered diet L. Mammary explants removed from the glands of rats initially offered diet L before a 24 h period of re-alimentation with diet H and incubated in either M199 or FBS incorporated thymidine at a rate 400 % greater than that of tissue taken from rats offered diet H from day 1 of lactation and incubated in similar media. As the tissues were incubated under similar conditions, this represents a genuine effect of dietary treatment; re-alimentation with a high protein concentration diet initiates mitosis. As the increase in mammary cell mass noted under similar dietary conditions

has previously been shown to be an effect of the associated energy intake arising on re-alimentation with a diet of high protein concentration and not the increase in protein intake (Chapter 4), this rapid increase in cellular proliferation may be ascribed to this increase in energy intake. The addition of foetal bovine serum to M199 had no effect on the proliferation rates of tissue removed from these rats when compared to tissue incubated in only M199, again indicating that the explants had maintained the necessary factors to undergo a rapid rate of cellular proliferation. However, rates of incorporation in tissue removed from these rats and incubated in D-PBS were significantly lower than those incubated in either M199 or FBS. Additionally, exogenous substrates were required to maintain such a high rate of cellular proliferation. Although this clearly demonstrates that mammary epithelial cells are capable of proliferating at a much faster rate than would be expected at this stage of lactation, the exact nutrient trigger is not known. However, this method is sensitive enough to allow the incubation of explants from rats offered a diet with a low protein to energy ratio in D-PBS with the addition of specific nutrients to determine this trigger.

The regression analysis of trial 3 suggested that the increase in mammary cell mass, observed when protein restricted rats were offered specific intakes of both protein and energy, was a result of the increased energy intake. It is possible that the explants from group LH are only responding to energy yielding nutrients provided by M199 but which are not present in D-PBS.

Feeding a diet of adequate protein concentration results in a significant increase in mammary cell mass over the first few days of lactation in mice (Brookreson and Turner, 1959; Knight and Peaker, 1982a, Shipman *et al.*, 1987), goats (Knight and Peaker, 1984b) and rats (DeSantiago *et al.*, 1991 and Chapters 2, 4). However, offering a diet of low protein to energy ratio during early lactation suppresses this increase in cell mass (Chapters 2 and 4). Unfortunately, data for [^{3}H]-thymidine incorporation in mammary explants taken from rats offered diet L for the first 7 days of lactation (group LL) was not obtained from this experiment. However,

the maintenance of the low but stable cell mass during early lactation would suggest that either both cell proliferation and death were high, or that both were low.

Cell death may be either 'natural' and part of normal cell turnover, termed apoptosis, or 'accidental', due to environmental perturbation, termed necrosis (Wylie *et al.*, 1980). Apoptosis is implicated in the steady-state kinetics of healthy tissues and it would be expected to be evident in mammary tissue, to a greater or lesser extent, irrespective of dietary treatment. Mammary secretory cell mass was assumed to be stable by day 7 of lactation in trial 1, but the [^{3}H] thymidine incorporation results taken from rats offered diet H in this experiment indicate a slow, but steady rate of proliferation. For cell mass to remain stable, the rate of cell death would logically have to be equal to the rate of proliferation. The electrophoretic gel (Figure 6.5) indicates, through the quantity of low molecular weight DNA present, that cells are dying in rats offered diet H for the first 6 days of lactation (Group H), however there is no sign of the laddering, characteristic of DNA cleaved as part of the apoptotic process. This is surprising as cell death through necrosis has not been observed as a mechanism for normal cell turnover (Wyllie, 1980). However a recent study by Lund *et al.*, (1996) also failed to identify apoptosis in lactating tissue, but it was clearly evident post weaning when the gland was rapidly involuting. It is possible that the current DNA labelling and electrophoresis techniques are masking the laddering effect which results from cleavage of DNA into fragments of low molecular weight with similar numbers of base pairs. Offering a diet L for both the first 6 (Figure 6.5) and 12 (Figure 6.4) days of lactation increased the mass of low molecular weight DNA present in the mammary tissue removed from the rats used in trial 1, indicative of a high rate of cell death. The absence of laddering in these samples would indicate that this cell death was through necrosis as opposed to apoptosis. Once again this is surprising as apoptosis is known to be triggered by a number of factors including increased levels of plasma glucocorticoid hormones (Wyllie, 1980) which are elevated during catabolism of endogenous protein (Tischler *et al.*, 1988). Offering a low protein diet has been shown to result in catabolism of the gastrocnemius muscle (Pine *et al.* 1994d) with a reduction in total carcass protein by both day 10 (Chapter 5) and day 12 (Pine *et al.* 1994a) of lactation. Cells dying

through natural, programmed cell death have been reported for murine mammary tissue during normal lactation and post weaning (Quarrie *et al.*, 1995), therefore it can only be suggested that apoptosis is difficult to identify in lactating tissue.

Transferring from a diet with a low protein to energy ratio to one with a higher protein to energy ratio after both 6 (LH) and 9 (LLH) days of lactation reduces the mass of low molecular weight DNA (Figure 6.5). This suggests that the rate of cell death has been reduced as a result of the increase in food intake associated with transferring to a diet of high protein to energy ratio; there was no evidence of apoptosis.

Extracting low molecular weight DNA and running it along an agar gel gives a good indication of the occurrence of cell death, but this method does not allow cell death to be accurately quantified. The morphology, as observed through the light microscope, of cells undergoing death has been extensively described (see Wyllie 1981). Common to both necrosis and apoptosis is the compaction of the cellular chromatin (pyknosis) which is readily identifiable through the light microscope. Apoptosis characteristically affects single cells rather than tracts of contiguous cells as is seen with necrosis (Searle *et al.*, 1982) and although large numbers of cells may be deleted over a short time period, the stromal-parenchymal organisation of the tissue remains intact (Wyllie, 1980). Although counting cells undergoing pyknosis is open to subjectivity, sufficient repeated counts reduces this effect. Tissue from rats offered diet L for the 7 day lactation period had a significantly elevated incidence of cells showing pyknosis when compared to those offered diet H. At this time, day 7 of lactation, the results from trial 1 would suggest that there would be no net change in either of these two epithelial cell populations. If the epithelial cells have an increased rate of death, there must also be an increased rate of proliferation, when compared to rats offered diet H, although this was not measured. It may be assumed that the cell death rate of rats offered diet L would be similar on days 6 and 7, in which case re-alimentation with a high protein to energy ratio diet rapidly reduced the number of cells undergoing pyknosis to a level significantly lower than those offered diet L and not different from those offered diet H from day 1.

The number of epithelial cells undergoing pyknosis per alveolus was relatively low in all dietary treatment groups and these cells had often separated from the epithelial monolayer, indicative of apoptosis and not necrosis.

In summary it may be concluded that a 24 h re-alimentation period with a diet of high protein to energy ratio after a 6 day period of protein restriction rapidly increased mammary epithelial cell proliferation and significantly reduced the rate of cell death in rats. Rats offered a low protein to energy ratio diet for the first 7 days of lactation showed significantly high rates of cell death when compared to those offered a high protein to energy ratio diet.

CHAPTER 7

DISCUSSION AND FURTHER WORK

7.1 INTRODUCTION

The primary objective of this thesis was to assess the sensitivity of the mammary gland, in terms of secretory cell number and activity, to both protein and energy intake during lactation. Trial 1 (Chapter 2) clearly showed that, in these terms the mammary gland of the rat was sensitive to nutrient intake. Whilst endeavoring to attribute the effects of increased nutrient intake on the mammary gland to either protein and/or energy intake, the suppression in food intake resulting from offering diets of low protein to energy ratio was also examined. In addition, milk is synthesised from substrates derived from both dietary and endogenous sources and it is therefore important to quantify the contribution of body reserves to the total nutrient demand, if lactation is to be studied holistically.

As a result, this thesis has two clear themes. The first one is the sensitivity of the lactating mammary gland to diets of different protein to energy ratio. The secondary theme is the lactating rat's response in terms of food intake and associated catabolism of body reserves to diets which are protein-energy imbalanced.

As each experiment has been discussed in detail at the end of the relevant chapter, the aim of this discussion is to draw the work together and discuss it in the context of lactational performance. As lactational performance is ultimately dependent upon the supply of nutrients to the mammary gland, whether they be of dietary or endogenous origin, the secondary theme will be discussed first.

7.2 RESPONSES TO DIETS OF LOW PROTEIN TO ENERGY RATIO

The results presented here show that lactating animals suckling a large litter, and offered a diet with a high protein to energy ratio, rapidly increase their voluntary food intake; their DM intake on day 10 of lactation being more than three times their intake on day 2. This was in contrast to rats offered a diet with a lower protein to energy ratio which showed a constrained daily intake (Figure 2.2). This response to diets of low protein to energy ratio has been reported previously for lactating rats (Naismith *et al.*, 1982; Friggens *et al.*, 1993; Pine *et al.*, 1994a,b,d) and it is

detrimental to lactational performance. As lactational performance is highly correlated with food intake, it is imperative to maximise the intake of a balanced diet if maximal milk yields, for the species or genetic strain are to be obtained.

In other circumstances, the reduced protein content of the diet would have been expected to cause an increase in total food intake in order to alleviate the deficit in dietary protein as is the case with growing pigs (Kyriazakis *et al.*, 1990). There have been a number of proposals as to why intakes in lactating animals are suppressed under these conditions, of which a number will be discussed.

The length, mass and functional capacity of the small intestine have been reported to increase during lactation (Hammond and Diamond, 1992, 1994). Hammond and Diamond (1994) suggested that food intake, and therefore lactational performance, in mice was limited by the small intestine's ability to undergo hypertrophy and thus absorb nutrients. If food intake was being restricted by the capacity of the small intestine, the lactating mice would have been expected to catabolise body reserves to supplement the nutrient shortfall, with a resultant loss of body mass as has been reported for rats (Pine *et al.*, 1994a,b,c; Chapters 2, 3, and 4) and cattle (Paquay *et al.*, 1972; Biddle *et al.*, 1975). In contrast, the mice used in their study gained body mass during lactation, suggesting small intestine capacity was not limiting food intake and another mechanism was regulating maximal lactational performance.

Rats offered diets of low protein concentration during lactation have been shown to undergo neither the liver nor gastro-intestinal hypertrophy observed in well-nourished rats (Pine *et al.*, 1994a). During periods of protein restriction, body organs are in competition for available amino acids and energy. It is generally accepted that the small intestine has a high protein turnover rate which is expensive in terms of energy as well as protein (Lobley *et al.*, 1980). Availability of substrates required for hypertrophy in the digestive tract may be further reduced during lactation by the active partitioning of nutrients towards the mammary gland (Bauman and Currie, 1980). Although the capacity of the small intestine would appear not to limit food intake when protein-energy balanced feeds are available, it may do so when lactational

demand is high and an imbalanced feed (*i.e.* with a low protein to energy ratio) is offered, as was the case with rats offered diet L and suckling a large litter. The sensitivity of the small intestine to protein restriction and re-alimentation was studied in trial 1 and the results are briefly presented here. The small intestines of rats used in trial 1 were removed immediately post mortem, their lengths recorded and dry masses determined after thoroughly washing the intestine in saline (150 mol l^{-1}) and freeze drying to a constant mass (Figures 7.1 and 7.2).

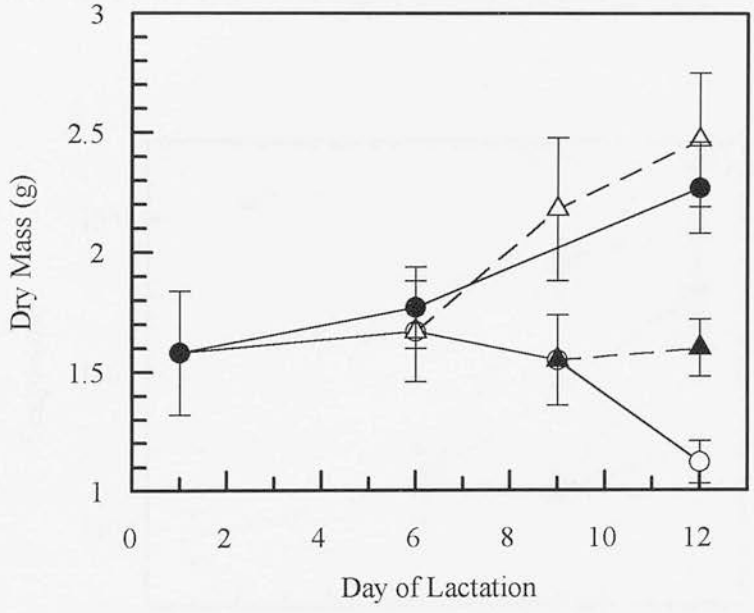

Figure 7.1 Small intestine dry mass of rats from trial 1 offered either a low (L)- or high (H)- protein diet for the first 12 days of lactation, or diet L with transfer to diet H after either 6 or 9 days lactation: (–○–), LLL; (–▲-), LLH; (–△-), LHH; (–●–), HHH. Values represent a mean and SEM, using dam day 1 gestation weight as a covariate.

The small intestines removed from rats offered a diet with a high protein concentration (group HHH) did increase both their dry masses and lengths ($P<0.05$) over the 12 days studied. This hypertrophy was not seen in rats offered a diet with a low protein concentration over the same period (group LLL). Rats offered diet L for both 6 (group LHH) and 9 (group LLH) days showed hypertrophy on re-alimentation

with diet H. Although there were no intermediate results between day 6 and 9 and also day 9 and 12 the hypertrophy was not likely to be either sufficient or rapid enough to explain the increase in voluntary food intake (Figure 2.2) and associated increase in lactational performance (Figure 2.3) observed in these rats.

These results would suggest that although lactational performance may be limited by food intake, food intake is not limited by the physical size of the small intestine. Recent work also concluded that the absorptive capacity of the intestines was not the ultimate limit to lactational ability and that the mass of the small intestine was highly correlated with daily food intake (Hammond *et al.*, 1996).

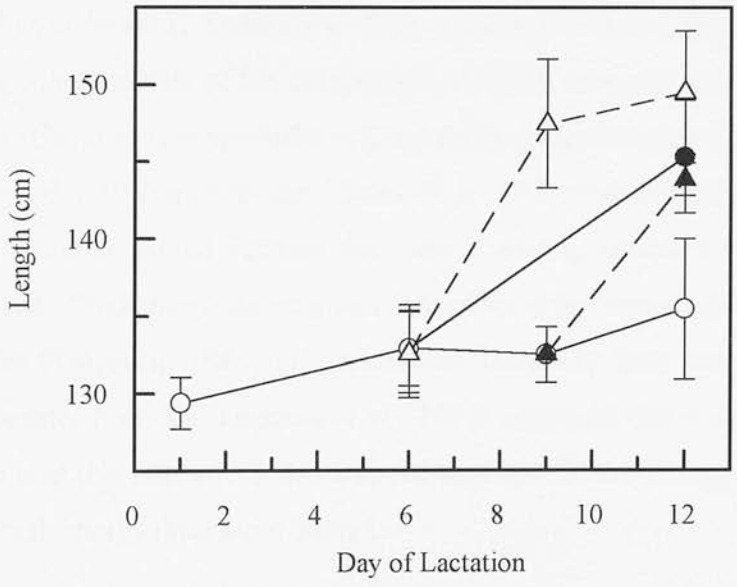

Figure 7.2 Small intestine length of rats from trial 1 offered either a low (L)- or high (H)- protein diet for the first 12 days of lactation, or diet L with transfer to diet H after either 6 or 9 days lactation: (–○–), LLL; (–▲-), LLH; (–△-), LHH; (–●–), HHH. Values represent a mean and SEM, using dam day 1 gestation weight as a covariate.

The daily food intakes of dams re-alimented with a diet of high protein concentration (diet H) after a period of protein restriction (groups LHH and LLH in trial 1) rapidly increased until they were comparable with those which had been

offered diet H from parturition. The rapidity of this increase in daily food intake would suggest that the constraint is also lifted quickly. A physical constraint, for example small intestine capacity, is unlikely to be responsive enough to explain this. The suppression in food intake seen when lactating rats are offered a diet with a low protein to high energy ratio must therefore be a consequence of another mechanism(s).

Increasing food intake of a diet with a low protein to energy ratio in excess of the intake seen for similar isoenergetic diets of high protein concentration in order to satisfy the protein requirements of lactation would, of course, increase the intake of energy yielding nutrients. The hormonal environment prevalent during early lactation has been shown to reduce the capability of adipose tissue to synthesise lipid whilst the ability of adipose tissue to undergo lipolysis increases (Marinchenko *et al.*, 1992). The compositional analysis of the carcasses, abdominal contents and skins from the rats in trial 3 (Chapter 5) supported this proposal as losses in adipose tissue between parturition and day 10 were between 14 and 40 g. Obligatory catabolism of body fat during early lactation would increase the energy yielding nutrients available to the lactating animal. These nutrients must be oxidised for either maintenance, lactation or thermogenesis (Trayhurn, 1985) or alternatively stored as body fat. Oxidation of nutrients generates heat, and Friggens *et al.* (1993) proposed that it was the female's incapacity to lose this heat which was constraining food intake during lactation when low protein-high energy diets were offered.

The food intakes of rats offered diet L (trial 1) were relatively variable; for example one rat only ate 64.6 g/12 d and another ate 248.6 g/12 d, with a mean intake of 162.6 (SEM 18.3) g/12 d. This variability would suggest that some rats were more able to consume a diet with a low protein to energy ratio than others. The fixed intake regimen of trial 3 allowed a distinction between those rats which were able to tolerate diets with a low protein to energy ratio between day 5 and 10 of lactation and those which were not.

No measurements of adipose tissue accretion during gestation or catabolism during early lactation were made for the rats used in trial 3. However, the liveweight

gains of rats not able to tolerate the diets with a low protein to energy ratio were greater (P=0.01) than those which were, 64.4 (SEM 5) g compared to 40.9 (SEM 6) g . Speculatively, it may be assumed that the animals gaining most body mass during gestation were also fatter at parturition. This may influence the response to nutrition during lactation, as found in dairy cows (Garnsworthy, 1988). As stated above, the hormonal status of the animal during lactation inhibits accretion of body fat and increases lipolysis (Marinchenko *et al.*, 1992). Catabolism of adipose tissue during this period would therefore be expected to be highly correlated with the animal's fatness at parturition. Fat animals would catabolise more adipose tissue that thin ones and this extra source of energy yielding nutrients, in conjunction with a diet of high energy to protein ratio may lead to a metabolic embarrassment. Under these conditions, it may be hypothesised that food intake would have to be restricted to the level at which all the energy yielding nutrients could be utilised for maintenance and lactation.

The energy intake of rats from trial 1 which were offered diet H *ad libitum* (group HHH) was 48 % more than those rats offered diet L *ad libitum* over the 12 day lactation period studied. In addition, rats offered diet H *ad libitum* between day 1 and 10 of lactation (trial 3) also lost a considerable (P<0.001) mass of adipose tissue (41.1 (SEM 5) g) over this time period so it may be concluded that energy yielding nutrients alone are not constraining intake of the low protein - high energy diet. By modelling the components of heat production and loss in lactating rats offered diets of different protein to energy ratio and housed at two different environmental temperatures, one standard (21 °C) and one low (11 °C) it was possible to estimate the point at which animals became heat stressed (Jessop, 1996b). This work clearly showed that lactating rats offered the diet with a low protein to energy ratio were not heat stressed at any time during early lactation, in contrast to the proposal of (Friggens *et al.*, 1993), whereas lactational performance would be limited by heat stress from day 6 of lactation in those offered a diet with a high protein to energy ratio kept at 21 °C (Jessop, 1996b).

Jessop (1996a) showed that the food intake constraint seen in animals offered the diet with a low protein to energy ratio could theoretically be lifted by supplying more protein through either an increase in the diet's protein to energy ratio or through an increase in the animal's rate of endogenous protein mobilisation. Increasing the dietary protein to energy ratio in mid lactation (day 5, 6 or 9) was shown to increase food intake in both trials 1 and 3, supporting this proposition. Addition of protein to the animal's nutrient pool allowed at least some of the surplus energy to be utilised for milk production. The more protein available, the more energy could be utilised and the higher the predicted food intakes, until limited by heat stress (Jessop, 1996b). Predicted intakes agreed very closely with observed ones from mid lactation (day 8), but were consistently higher than observed over the first eight days of lactation, suggesting that during early lactation there is a further constraint on food intake of a diet with low protein to energy ratio.

During early lactation, skeletal muscle (gastrocnemius) protein has been shown to be catabolised in response to dietary protein restriction (Pine *et al.*, 1994d) with the bulk of the loss being achieved by day 8 of lactation. This loss of protein was associated with a dramatic increase in the fractional degradation rate (Pine *et al.*,1994d). Haemoglobin is also catabolised during periods of dietary protein restriction (Nasset and Gatewood, 1954), which has been shown to lead to anaemia in protein-energy malnourished humans (Alleyne *et al.*, 1974). Catabolism of these proteins has been shown to elevate the levels of plasma histidine in relation to the other amino acids as discussed in Chapter 3. It has been proposed that this histidine is then transported across the blood-brain barrier where it is converted to the neurotransmitter histamine which binds to receptors on the hypothalamus to reduce voluntary food intake (Orthen-Gambill, 1987; Mercer *et al.*, 1989; Mercer *et al.*, 1994). If this proposal is correct, administration with a drug which is a competitive inhibitor to histamine, for example cyproheptadine, would be expected to increase food intakes; this was shown to be true for growing rats (Mercer *et al.*, 1994). Lactating rats offered the diet with a low protein to energy ratio and injected with cyproheptadine showed improved daily food intakes, to levels similar to the theoretical ones predicted by Jessop (1996a) when compared with rats offered the

same diet and injected with saline (Chapter 3). After day 8 of lactation, intakes of the rats treated with cyproheptadine fell to levels previously seen for rats offered the diet with a low protein to energy ratio, and to the levels predicted by the model of Jessop (1996a). At this time, mobilisation of skeletal protein would be low (Pine *et al.*, 1994d) and plasma histidine would therefore be expected to return to normal values, although this was not assayed, and the drug would not be expected to have an effect on food intake (Figure 3.1).

In summary, this work would indicate that two possible mechanisms explain why food intake is not increased when lactating rats are offered a diet with a low protein to energy ratio, in contrast to the work on growing pigs (Kyriazakis *et al.*, 1990). Firstly, dietary protein restriction is likely to lead to rapid catabolism of muscle carnosine and haemoglobin, both rich in histidine which triggers an histaminergic response, suppressing food intake for the initial 8 days of lactation. Secondly, the hormonal milieu prevalent during early lactation, prevents the accretion of surplus energy yielding nutrients which would arise if food intake was increased to maintain protein intake. Re-alimentation with a diet of high protein concentration would prevent muscle protein mobilisation and therefore reduce brain histamine concentration and also provide more amino acids in order to utilise the surplus energy yielding substrates for milk production. Both these mechanisms would be expected to be lifted rapidly and could easily explain the rapidity of the increase in food intake seen in the experimental work described in this thesis.

The source of nutrients available to the mammary gland for milk synthesis may be from exogenous (dietary) origin and/or from the mobilisation of body stores. The work of Marinchenko *et al.* (1992) would suggest that mobilisation of adipose stores during early lactation is obligatory due to the prevailing hormonal status of the animal, assuming adequate accretion of adipose tissue during lactation. The data presented in Chapter 5 would support this as rats from all dietary treatment groups lost ($P<0.001$) body fat (the sum of carcass, organ and skin fat) over the period studied. It was argued above that this was detrimental to food intake, and as a consequence lactational ability, unless sufficient protein was available to enable the surplus to be

used for milk synthesis. When such animals are maintained in a thermoneutral environment and sufficient protein is available, lactational performance is only limited by heat stress (Jessop, 1996b). Consequently, it could be argued that lactational mammary development of rats well-nourished during gestation and offered high energy diets during lactation (as all the rats in this work were) is never constrained through lack of energy yielding nutrients. This has been confirmed through the construction of crude nutrient balances. However, the multivariate regression analysis described in Chapter 4 clearly showed that the suppression in mammary gland development seen in rats which had been offered a diet with a low protein to energy ratio was lifted as a result of an increase in non-crude protein gross energy (NCPGE) intake more than crude protein (CP) intake. This would suggest that the mammary gland is energy limited. This is surprising as the concentration of milk lipid was higher ($P<0.01$) in the milk taken on day 10 from rats offered the low protein-high energy diets when compared to those offered diets with higher protein to energy ratios in this study (Chapter 4) and also in the study of Pine *et al.* (1994c). These authors proposed that this increase in milk lipid concentration was a means of dissipating, at least in part, the surplus energy-yielding nutrients arising as a consequence of offering protein-energy imbalanced diets. If mammary gland development, in terms of both the secretory cell population and the activity of these cells, is energy limited, what would be the benefit of increasing the lipid content of the milk?

The mother apportions a high priority to the survival of her young, but offering diets of low protein concentration have been shown to impair milk production (Pine *et al.*, 1994a,b,c; Chapters 2, 3 and 4). It is possible that by increasing the milk's lipid content the litters are able to obtain sufficient energy to survive, although daily growth rates are low. Presumably the regulatory aspects of mammary development are under the control of the endocrine, paracrine and autocrine systems and it is possible that energy yielding nutrients which have arisen from endogenous reserves do not trigger cellular proliferation whereas dietary supplies do. Speculatively, energy yielding nutrients of dietary origin may increase

levels of plasma IGF-I which is a known mitogen (Buttle and Lin, 1991; McGrath *et al.*, 1991; Forsyth, 1996) whereas substrates from endogenous sources do not.

Many studies have suggested that reserves of endogenous protein, which may provide up to 250 g/kg body protein in both lactating rats (Allison and Wannemacher, 1965; Pine *et al.*, 1994a) and dairy cows (Botts *et al.*, 1979), are utilised by well-nourished rodents suckling large litters (Kanto and Clawson, 1980; Sainz *et al.*, 1986a,b; Taylor *et al.*, 1986). The results of trial 3 (Chapter 5) did not support this and were in agreement with those of Pine *et al.*, (1994a) and Friggens (1991). Dams offered diets with a high protein to energy ratio increased their food intake in order to maintain the high lactational performance required to suckle a large litter, without net mobilisation of body protein (Table 5.2). Taylor *et al.* (1986) argued that animals not mobilising endogenous protein were of lower genetic potential in terms of lactational ability. While it is possible that the dams offered the 215 g CP/kg DM diet (diet H) in this study were of a lower potential, the daily weight gains of the standardised litters were in excess of those from the study of Taylor *et al.* (1986), indicating otherwise.

In contrast to the lactating females offered diets with a high protein to energy ratio, those offered diets with a lower ratio mobilised a significant mass of endogenous protein. Nitrogen balance during lactation has been shown to be closely related to the insulin to cortisol ratio, insulin having an anabolic effect and cortisol a catabolic one in rats (Kliewer and Rasmussen, 1987) and in humans (Motil *et al.*, 1994). It is widely accepted that endogenous protein, in conjunction with that of dietary origin is partitioned towards the mammary gland and splanchnic tissues at the expense of other tissues. Even with active partitioning towards specific organs, protein was lost from all tissues assayed when diets of low protein to energy ratio were offered (Pine *et al.*, 1994a,d; Chapters 2, 5); presumably in an attempt to maintain milk volume and the protein concentration of milk. The multivariate regression analysis on the data for total body (carcass, skin and organ) protein loss between day 5 and 10 of lactation (Chapter 5) showed that after a period of protein restriction, increasing dietary crude protein intake by 1 g spared the loss of body protein by only 0.19 g (P=0.041). This is further support that maintaining milk

protein concentration is ascribed a high priority by the animal, as opposed to skeletal protein repletion. In addition, this low coefficient supports the suggestion that food intake of diets with a low protein to energy ratio is restricted during mid lactation by the inability to dissipate surplus energy yielding nutrients.

7.3 THE RESPONSE OF THE MAMMARY GLAND

In all the studies presented, the weight gain of a standardised litter has been used as an index of milk production. This is not an accurate predictor of milk yield since no consideration is given to pup maintenance requirements or possible variations in nutrient supply that result from deviations in milk composition (Romero *et al.*, 1975; Pine *et al.*, 1994c). Even so, it is generally accepted as a good qualitative index for lactational performance.

As has been continually stressed throughout this thesis, maximal lactational ability depends on the maximum possible number of secretory cells working at full potential. An important aspect of milk secretion is the relationship between lactational performance, secretory cell number and cellular activity. Post partum proliferation of cells has been estimated to account for only a small proportion of the increase in milk yield prior to peak yield in the goat; cellular activity accounting for the majority of the increase (Knight and Peaker, 1984b). In contrast, the increasing secretory cell proliferation has been shown to account for approximately 66 % of the increase in yield (yield was estimated using the mammary gland weight difference technique) between parturition and day 7 of lactation in rats (Knight *et al.*, 1984). In the studies reported in this thesis, daily litter weight gains of rats offered diet H (trial 1, Chapter 2) increased from 11.8 (SEM 1.12) g/d on day 2 of lactation to 20.5 (SEM 1.34) g/d on day 6, an increase of 74 %. Total mammary DNA (DNA_t) increased from 11.1 (SEM 1.48) mg to 18.7 (SEM 1.87) mg, an increase of 68 %. Therefore the increase in cell number can be estimated to account for 92 % ($68 \times 100/74 = 92$ %) of the increase in yield during this time. This is higher than that estimated by Knight *et al.* (1984) and is probably a result of the different methods used to estimate milk yield. As previously stated, daily litter weight gain is not a good predictor of milk

yield as the maintenance costs of the litter are not considered (Romero *et al.*, 1975; Pine *et al.*, 1994c). The daily milk yield of the rats offered diet H between day 1 and 6 of lactation was therfore estimated using the equations of Pine *et al.* (1994c) which take into account the maintenance costs of the litter. On day 2 of lactation dams would be synthesising 23.8 (SEM 1.9) g of milk per day and by day 6 they are estimated to be producing 44.4 (SEM 2.4) g per day, an increase of 87 %. The increase in secretory cell number is now estimated to account for 78 % of the increase in milk yield between day 1 and 6 of lactation. This confirms that daily litter weight gain, as an indication to lactational performance underestimates milk yield and would also suggest that the mammary gland weight difference technique is an overestimate. Most importantly, this emphasises the importance of mammary growth during early lactation. Although increases in the secretory cell population during this time are thought to be smaller in ruminants (Knight and Wilde (1987), maximisation of secretory cell proliferation through optimum nutritrition may have enormous implications for the dairy industry. Offering a diet with a high protein to energy ratio between day 6 and 9 of lactation after a period of severe protein restriction (group LH; trial 1) increased daily litter weight gain by 500 % (4.0 (SEM 1.18) to 24.2 (SEM 2.20) g/d). However, this is obviously a gross overestimate of the actual increase in milk yield, clearly demonstrating the importance of considering the maintenance costs of the litter. Using the equations of Pine *et al.* (1994c) the milk yield of group LH (trial 1) increased from 22.8 (SEM 1.6) g/d on day 6 to 56.0 (SEM 3.2) g/d on day 9, an increase of 146 %. Total mammary DNA increased from 10.3 (SEM 0.58) to 16.2 (SEM 1.93) mg over the same time period and therefore the contribution of DNA_t to lactational performance was only 39 %, the difference assumed to be due to an increase in cellular activity.

Similar calculations cannot easily be made for the data of trial 4 (Chapter 4) between day 5 and 10 of lactation; however the regression analysis would suggest that cellular activity (lactose synthetase activity per mg DNA) made the more significant contribution to the increase in lactational performance observed on re-alimentation with dietary energy, in support of the above calculation (Table 7.1).

Table 7.1 Coefficients, standard errors and probabilities for the contributions of the increase in total mammary DNA (ΔDNA_t; mg) and lactose synthetase activity (Δactivity; nmoles lactose formed / mg DNA / min) between day 5 and 10 of lactation (trial 3) to increase in daily weight gain of standardised litters (g/5 d) following multivariate regression analysis. The following model was used: Δlitter weight (g/5 d) = $\alpha + (\beta \times \Delta DNA_t$; mg) + ($\chi \times \Delta$activity; nmoles lactose formed / mg DNA / min). There was no significant interaction between DNA_t and activity.

	Adj. r^2 (%)	Estimate	SE	*P*
	44.0			<0.001
α		37.1	1.94	<0.001
β		0.4	0.15	0.471
χ		0.3	0.01	<0.001

This huge increase in cellular activity on re-alimentation with diets of higher protein to energy ratio implies that the mammary secretory cells of rats offered diet L may not be fully differentiated when lactation is well established, if diets of low protein to energy ratio are offered.

The rapidity of the increase in secretory cell number and activity of these epithelial cells seen when diets with a high protein to energy ratio were offered after a period of protein restriction (trial 1 and 3) forms the crux of this thesis. This remarkable phenomenon poses some interesting questions. Firstly, was this an effect of the increase in intake of crude protein or an increase in the dietary energy intake which resulted from the improved food intake? Secondly, was this increase in total mammary cell mass a consequence of an increased rate of cell division, a decreased rate of cell death or a function of both? The answer to the latter was found to be a change in the rates of both, cell proliferation and cell death (Chapter 6) demonstrating the need to consider the effects of cell loss on milk yield as well as the effects of cell proliferation.

Since secretory cell number is one determinant of milk yield, and it would appear physiologically possible to increase cellular proliferation and decrease cell loss on day 7, when lactation is thought to be fully established, (Chapter 6), the factors controlling these mechanisms are of crucial importance. Lactating dams offered a diet

with a low protein to energy ratio during early lactation do not show the increase in cell mass associated with well-nourished dams (Chapter 2 and 4). It was unfortunate that rates of [^{3}H] thymidine incorporation were not obtained from animals offered diet L (Chapter 6). So it is only possible to speculate that the secretory cell proliferation rates of these animals were still elevated on day 7 of lactation, when compared to well-nourished rats at this time, as rates of cell loss were significantly higher (Figure 6.6) but cell mass remained constant. This would imply that it is the high rate of cell death which is preventing the increase in mammary cell mass seen in well-nourished rats during early lactation. If this were the case, the question arises as to the mechanism by which elevated rates of cell division ceases after the first few days of lactation in well-nourished but not in protein-energy malnourished rats. The nature of the signalling pathway controlling this mechanism has yet to be elucidated but it is known to involve a complicated orchestration of endocrine, paracrine and autocrine interactions (Taketani and Oka, 1996; Wilde and Burgoyne, 1990; Thoradson, *et al.*, 1992; Ip *et al.*, 1992; Thompson *et al.*, 1992; Miettinen *et al.*, 1994). Current thoughts on this complex interaction of control mechanisms have recently been described by a number of authors (Medina, 1996; Wilde and Hurley, 1996; Jaggi *et al.*, 1996; Forsyth, 1996) and as there are no conclusive results, the topic will not be discussed further here.

Although no apoptosis was detected by the methods of Tilly and Hseuh (1993) during the work of trial 4, in agreement with Lund *et al.* (1996), cell death was shown to be occurring in tissue from all dietary treatment groups (Figure 6.6) and the morphological characteristics of cell death were seen through the light microscope. The concept that the epithelial cells from well-nourished animals are undergoing cell turnover was further established by the low but steady rate of [^{3}H] thymidine uptake seen in trial 4 (Figure 6.3). The cells degenerating as part of this natural process would be expected to do so by apoptosis and not necrosis. Epithelial cells from rats offered diet L were being lost at a faster rate than those offered diet H, or those transferred from diet L to diet H on day 6 of lactation (Figure 6.6). Again, it is not known for certain whether this loss of cells was by apoptosis or not, but the histology would suggest that it was. In addition, cell loss through programmed cell death has

been reported in murine mammary tissue, both pre- and post-weaning using the nick-end labelling technique (Quarrie *et al.*, 1995) which would suggest that cells are lost as part of normal cell turnover, through apoptosis. Nick end-labelling may be a more sensitive technique for detecting apoptosis in mammary tissue. If, as speculated above, this increased rate of cell death is preventing the increase in cell mass seen during early lactation, it is pertinent to understand the mechanism(s) involved.

Epithelial cell growth and integrity depend on the interactions between themselves and the extra cellular matrix (ECM) (Streuli *et al.*, 1991; Bordeaux *et al.*, 1995; Cunha and Hom, 1996). Bordeaux *et al.* (1995) suggested that these interactions act through integrin receptors on the cell membrane initiating and maintaining the differentiated state of mammary epithelial cells and also suppressing apoptosis. Disruption of ECM, and as a result the integrin receptors, with proteases triggered the expression of genes that resulted in programmed cell death (Talhouk *et al.*, 1991). Two classes of enzymes have been implicated in a proteolytic cascade of the ECM degradation: metalloproteinases and plasminogen activators. The metalloproteinases appear to be the rate-limiting enzymes (Talhouk *et al.*, 1991) but the plasminogen activators are produced in quantity within the mammary gland and activate plasmin from its inactive precursor, plasminogen (Ossowski, *et al.*, 1979). Plasminogen activator activity, and hence mammary plasmin concentration, have been shown to be influenced by the IGF-I to insulin ratio (Politis *et al.*, 1990) so that as this ratio increases, the concentration and activity of plasmin increases. This would lead to the degradation of the ECM within the gland, loss of integrin receptors and loss of epithelial cells through apoptosis. The IGF-I to insulin ratio increases towards the end of lactation as serum insulin concentration increases (Vernon, 1988). The IGF-I to insulin ratio would also be affected by nutrient supply, diets with a low protein to energy ratio increasing the ratio favouring plasmin activity and loss of secretory cells. This would be a plausible mechanism worthy of further experimentation. If this were to be the case, manipulating the diet to maintain a low IGF-I to insulin ratio would favour secretory cell proliferation reducing the rate of cell loss through apoptosis. As loss of secretory cells is the principle cause of the post peak decline in milk yield in goats and a major cause in rodents (Knight, 1989)

maintaining the secretory cell population for a longer period of time would result in a greater total milk yield.

This discussion has attempted to join the work of the four experimental trials together, and place it in context with other literature in order to further our understanding of lactation which is a process involving the whole animal and not solely the mammary gland. While the study has improved our understanding of the mammary gland's response to diets of differing energy and protein content and also helped explain why lactating rodents offered diets of low protein to energy ratio paradoxically suppress their food intake, a number of additional questions remain to be answered.

7.4 FURTHER WORK

1) Determination of the rate of mammary cell proliferation of rats offered a diet with a low protein to energy ratio during early lactaion.

The work described in this thesis would suggest that the rate of mammary cell proliferation in rats offered a diet of low protein to energy ratio was high when compared to the rate of [^{3}H] thymidine uptake in cells taken from well-nourished rats on day 7 of lactation. However, this is still to be established.

2) Elucidation of the mechanisms controlling mammary cell turnover.

Although the mechanisms controlling mammary epithelial cell proliferation are undoubtedly complex, the *in vitro* explant culture model developed in trial 4 and described in Chapter 6, would provide a suitable method to measure cellular prolferation. The developed method may also be used to investigate how nutrient triggers, acting through endocrine, paracrine and autocrine systems, affect the rates of cell proliferation and differentiation. Further work is required to recognise apoptosis. The molecular mechanisms of apoptosis are poorly understood, however, in time it may be possible to characterise programmed cell death by other means. Several transcription factors have been implicated in the control of apoptosis, for example

Myc/Max, c-Fos, c-Jun, p53, E2F, and Nur-77. It may be possible to identify programmed cell death more accurately by assaying for these transcription factors.

3) Investigation of the effects of dietary protein to energy ratio on rates of mammary cell proliferation *in vivo*.

In contrast to other cell culture systems explant (and whole organ) culture models allow the normal glandular architecture to remain intact. Specifically, the mammary epithelial cells remain within their normal stromal environment allowing stromal-epithelium interactions to occur. However, these *in vitro* systems are still likely to behave in a different fashion to the *in vivo* situation. If the effects of dietary protein and energy on mammary development are to be studied definitively they should be repeated *in vivo*. However, the cost of radioisotope ([^{3}H] thymidine) would be high as relatively large doses would be required to ensure sufficient uptake by the mammary tissue to allow differences to be quantified.

4) The influence of adipose stores on food intake.

This work found a plausible explanation as to why lactating rodents offered diets of low protein to energy ratio suppress food intake, in contrast to the response shown by growing pigs (Kyriazakis *et al.*, 1990). However, this hypothesis is still to be elucidated for rodents and, of more economic importance for lactating farm animals.

This hypothesis could be tested by challenging animals which were both fat and thin at parturition with diets of low protein to energy ratio during early lactation. The thin animals would be expected to have elevated food intakes, and show an improved lactational performance when compared to the fat ones offered the same protein-energy imbalanced diets.

CONCLUSIONS

Offering a diet with a low protein to energy ratio (diet L) to rats suckling a large litter during early lactation (day 1-12) led to a reduced food intake when compared to rats offered a diet with a higher one (diet H). Although the two diets were isoenergetic, rats offered diet H ate 48 % more over the 12 day period. Their energy intake over this period was therefore 48 % higher than rats offered diet L and their protein intake 78 % higher.

This reduced intake of both protein and energy resulted in a reduced lactational performance, which was a result of mammary underdevelopment in terms of both secretory cell number and activity.

Re-alimentaton with a diet of high protein to energy ratio initiated an increase in mammary secretory cell mass and cellular activity, even after 9 days of protein restriction. There was a resultant increase in lactational performance.

Both the increase in cell mass and activity were largely a consequence of the increase in energy intake rather than the increase in crude protein intake. However, the consequential increase in lactational performance was a function of both dietary energy and protein intakes.

The mammary secretory cell proliferation rate, determined by *in vitro* [^{3}H] thymidine incorporation, of rats offered diet L for 6 days before being offered diet H for 24 h was significantly higher than the rate of cells removed from rats offered solely diet H from parturition. Similar data for rats offered solely diet L were not collected. Epithelial cell loss was significantly greater from rats offered diet L from parturition to day 7 of lactation when compared to rats offered solely diet H or diet L followed by diet H on day 6. There was no significant difference between the rates of cell death from animals offered solely diet H and those offered diet L and then diet H.

Suppression of food intake arising as a result of offering lactating rats diets with a low protein to energy ratio was found to be lifted by daily injections of the anti-histamine antagonist, cyproheptadine for the first 8 days of lactation only. Subsequently, daily food intakes returned to levels normally seen in rats offered this

diet at this stage of lactation. Pregnant animals which acquired the most adipose tissue during lactation were least likely to be able to consume a diet with a low protein to energy ratio after day 8 of lactation.

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APPENDIX I

MATERIALS AND METHODS

A1.1 ANIMALS.

In all experiments specific pathogen free Sprague-Dawley rats (B&K Universal Ltd., Hull) were used. All breeding females arrived with a body weight of 300 (SEM 5) g on arrival and had had one previous litter only. All males were previously experienced. On arrival, all females were housed either individually or in pairs for a period of at least 14 days to allow complete adjustment to their new environment. At the appropriate time, females were placed with a proven male breeder in a wire bottomed cage (N&K Plastic Cages, Ltd. Kent). The morning on which mating was confirmed, through the presence of vaginal plugs. was designated day 1 of gestation and the dam was returned to a solid-bottomed plastic cage for the remainder of the trial. The male was allowed a minimum of two days, for recovery, before being introduced to another female; this process was continued until all females had apparently conceived. If no vaginal plug was sighted after one week with the male, the dam was assumed to be pregnant and returned to a solid bottomed cage. Females falsely assumed pregnant were returned to a different male for a second attempt at conception, if this was again unsuccessful the female was considered infertile and removed from the trial.

Table i Animal numbers, conception rates and litter sizes for the four rat trials.

	Trial 1	Trial 2	Trial 3	Trial 4
Number of Dams	72	25	83	31
Number of Weanlings		36		
Number of Males	20	7	20	5
Conception Rate (%)	94	90	96	91
Useful Dams (%)*	82	76	86	81
Useful Weanlings*		100		
Mean litter Size†	12.0	14.0	12.8	12.5

* Useful animals were those which completed the experimental period and whose results were included in the analysis of data.

† Number of pups per dam born alive.

A1.2 Environmental Conditions.

All animals were caged either individually or in pairs in solid-bottomed plastic cages (45×28×20cm) (N&K Plastic Cages, Ltd. Kent) with dust-free sawdust for bedding (BN&S Ltd., Edinburgh) before mating. After mating all animals were caged individually until the end of the experiment. Each trial had 12 hour light/dark photoperiods, with the light period between 07:00 - 19:00 h in trial 1 and trial 4; 06:00 - 18:00 h in trial 2 and 06:30 - 18:30 trial 3. A radio-timer was preset to come on 15 minutes after the lights and switch off 15 minutes before the lights and entry to this room was not effected until after this time.

The animal room was maintained at approximately 21°C and relative humidity 55% (Table ii).

Table ii. Animal room temperature (°C) and relative humidity (%) values (Mean with (SEM)) for the four rat trials. All readings taken on entry to the room in the morning.

	Trial 1	Trial 2	Trial 3	Trial 4
Mean Min. Temp. (°C)	19.3 (0.1)	19.6 (0.1)	20.2 (0.1)	19.7 (0.1)
Mean Max. Temp.	22.7 (0.1)	22.3 (0.1)	22.3 (0.1)	22.7 (0.1)
Mean Rel. Humidity (%)	52.6 (0.5)	54.3 (0.6)	54.9 (0.6)	58.2 (0.5)

A1.3 Female Handling.

All dams were handled daily from the time of their arrival until parturition in order to minimise the impact of such disturbances during lactation on lactational performance and pup mortality. Since rats and in particular lactating dams are inherently nervous, animals were always approached with a hand clothed in a latex examination glove to minimise smell variation and allowed a brief period of recognition before being disturbed. Litters were handled with the dam out of the cage and they were returned to their original position in the bedding before the dam was returned.

Due to natural variation in litter size, all litters were standardised to 12 pups on the morning following parturition, where possible. Cross-fostering was achieved by gently rubbing the fostered pup, born on the same day, in first the recipient dam's bedding and secondly with the natural litter. When it was not possible for dams with a natural litter size of less than 12 to receive foster pups born on the same day, she was removed from the trial.

A1.4 Feed Ingredients, Formulation and Manufacture.

This thesis examines the effects of dietary protein and energy on mammary development and lactational performance. It was therefore essential that all diets were formulated to a known crude protein and gross energy concentration. To achieve this, reductions in energy supplied as protein were replaced by increasing the carbohydrate (CHO) and fat contents in a fixed ratio (2.3 : 1) which was determined from their gross energy (GE: kJ/kg DM) contents:

$$\text{Proportion CHO} = \frac{(GE_{\text{Protein}} - GE_{\text{Fat}})}{(GE_{\text{CHO}} - GE_{\text{Fat}})}$$

$$\text{Proportion Fat} = 1 - (\text{Proportion CHO})$$

The constituent gross energies were determined by bomb calorimetry and were as follows: Protein 23.62 kJ/g DM; CHO 16.71 kJ/g DM and Fat 39.63 kJ/g DM.

The protein source was casein powder (110 mesh) (Bacarel & Co. Ltd., Kent) supplemented with DL-methionine (99/1 w/w). The carbohydrate was supplied by a 2:1 (w/w) mixture of starch (BDH, Poole) and sucrose (commercially available). The dietary fat source was corn oil (commercially available).

Once the proportions of protein, fat and carbohydrate had been determined the diet formulation was completed with the addition of vitamin and mineral supplements

(ICN, Ohio, USA) to meet the requirements of the lactating rat (NRC, 1978), (Table iii) antioxidant (butylated hydroxy toluene) and emulsifier (egg lecithin; Fisons, Loughborough). In addition, a fixed mass of corn flour (commercially available) was added to diets H and L, this was to maintain consistency with the work of Pine, 1993 so data comparisons could be made. All diets were formulated on a dry matter basis and the diet compositions are given in the relevant chapters.

Table iii Vitamin and Mineral Mixes supplied by ICN, Ohio,USA.

Vitamin Mix (mg/kg)		**Mineral Mix (g/kg)**	
Thiamine HCl (B_1)	600	Calcium Phosphate ($CaHPO_4$)	500
Riboflavin (B_2)	600	Sodium Chloride (NaCl)	74
Pyridoxine HCl (B_6)	700	Potassium Citrate (Monhydrate)	220
Nicotinic Acid (Niacin)	3000	Potassium Sulphate (K_2SO_4)	52
D-Calcium Pantothenate	1600	Magnesium Oxide (MgO)	24
Folic Acid	20	Manganous Carbonate	3.5
D-Biotin	20	Ferric Citrate (16-17% Fe)	6
Cyanocobalamin (B_{12})	1	Zinc Carbonate (70%ZnO)	1.6
Retinyl Palmitate (A) 250 i.u/mg	1600	Copper Carbonate (53-55% Cu)	0.3
dl-Tcopherol Acetate (E) 250 i.u/g	20000	Potassium Iodate (KIO_3)	0.01
Cholecalciferol (D_3) 400 i.u/mg	2.5	Sodium Selenite ($Na_2SeO_3.5H_2O$)	0.01
Menaquinone (K)	50	Chromium Potassium Sulphate	0.55

Filler was finely powdered Sucrose
In addition Vitamin B_{12} (40 μg/kg DM) and choline chloride (6.7 g/kg DM) were added.

As accurate measurements of feed intake were of paramount importance to all the afore-described trials, it was necessary to offer a food which was too soft for the animals to remove lumps from the container, but firm enough to prevent losses if the container was tipped. This was achieved by adding water to the diets, the amount added varying between diets, due to the different oil contents. Feeds were mixed in 3

kg batches using a commercial mixer and then stored (-15 °C) in sealed, plastic containers each holding approximately 1 kg.

The soft consistency of these diets necessitated the provision of plastic chew rings in order to allow the natural trimming of teeth.

A1.5 Daily Measurements.

During each trial all female rats were weighed daily, and during lactation litter weight changes were also recorded daily. Dam and litter weights were recorded using a Sartorius balance (2260 DBS) programmed to issue the mean of 10 separate readings (2 decimal places (d.p)) to minimise the effect of animal movement. The diet H described above was introduced to all animals from the first day of gestation in all four trials. Weighed samples of fresh food were offered daily in pre-weighed, 120 ml Beatson jars, which were placed into a plastic jar holder with a large flat base, to minimise the risk of the jar tipping.

All daily measurements were taken at a similar time each morning, starting within 1 hour of the light period.

A1.6 Carcass and Tissue Analysis

At the appropriate time during each experiment, groups of females were slaughtered for subsequent analysis. In all trials animals were culled by decapitation using a hand-operated guillotine. Following slaughter, the bodies of dams used for carcass analysis (trial 1 and trial 3) were dissected, the skins, entire mammary gland, and organs of the abdominal and thoracic cavities being removed and weighed individually before being sealed in separate plastic bags and stored at -15 °C. The small intestine was dissected from the stomach and the large intestine, and its unstretched length recorded. It was then split longitudinally and gently rinsed in saline (150 mol l^{-1}), before being stored at -15 °C. In addition, the tail was removed, but no analysis on either the head or the tail was carried out. No carcass analysis was performed on animals in trial 2 The liver was however removed in its entirety

following slaughter, and a portion was taken in order to assay for specific activity of the enzyme, liver histidine ammonia lyase (EC 4.3.1.3), A1.9. Only the analysis documented in Chapter 6 was performed on animals in trial 4.

All chemicals were obtained from Sigma Chemicals, Poole, U.K. unless otherwise stated.

A1.6.1 Carcass and organ analysis

The dry matter content of the frozen tissue sample, (carcass, abdominal and thoracic organs) was determined by freeze drying to a constant mass (Edwards Pirini 501 freeze dryer). The samples were milled (Retsch ultra-centrifugal mill) in a centrifugal mill designed to prevent overheating of the sample with consequent loss of fat. The milled samples were freeze dried for a further 24 h to ensure any moisture collected during the milling procedure was removed and samples were stored with dessicant at -15 °C in sealed bags. All subsequent analysis was performed on samples in duplicate. At the time of analysis approximately 5 g of sample was placed in a dry, pre-weighed glass crucible at 100 °C for 24 h before re-weighing. Any error due to moisture collected during storage could thus be corrected, although very few discrepancies were found.

A.1.6.1.1 Nitrogen and Crude Protein

Freeze dried carcass and organ samples were analysed for total nitrogen content using the micro-Kjeldahl technique.

1) Approximately 0.20 g was accurately (4 d.p.) weighed into a Kjeldahl flask and ~1 g reaction catalyst ($CuSO_4$:K_2SO_4 (1:3 w/w)) was added, as were 5 cm^3 conc. H_2SO_4 and anti-bumping granules; this was thoroughly mixed.

2) Each sample was digested on a heat mantle for approximately 7 h. During digestion the organic nitrogen was converted to ammonium sulphate.

3) The contents of the Kjeldahl flask were washed into a 100 ml volumetric flask and made up to volume with distilled water.

4) A 5 cm^3 aliquot plus 5 cm^3 5 mol l^{-1} NaOH was distilled using a Hoskin's distillation unit. Here, the ammonia bound as ammonium sulphate is released by steam distillation and collected by 0.16 mol l^{-1} H_3BO_3 in a conical flask.

5) The mass nitrogen was then titrated against a known molarity of HCl (M/140)

The acid was standardised against a solution of $(NH_4)_2SO_4$ with a known nitrogen content to determine the exact molarity of HCl.

From this standardisation it may be assumed that 1 ml (M/140) HCl ≡ 0.1 mg N.

Therefore	Titre (cm^3) × 0.1	≡	mg N in conical flask - (A)
	((A) × 20) / 1000	≡	g N in 100 cm^3 volumetric flask - (B)
	(B) × 6.25	≡	g crude protein (CP) in sample*

* Assumes nitrogen content of protein to be 16%.

A.1.6.1.2 Gross Energy and Fat Content

The fat content of both milled carcass and organs was estimated from the gross energy value obtained using a Gallenkamp, adiabatic bomb calorimeter. The energy liberated from a known sample mass is determined from the temperature rise of a known mass of water, and total heat capacity of the steel bomb in the following manner:

$$\text{Gross Energy (kJ / g DM)} = \frac{((\Delta t \times HC) - c)}{m}$$

where: Δt temperature rise (°C)
HC heat capacity of bomb (J/°C)
c correction factor for heat gain (J)
m sample mass (g)

Tissue fat content was calculated from the equation of Pine *et al.* (1994):

$$\text{Tissue Fat (g/g DM)} = \frac{GE - (23.6 \times CP)}{39.6}$$

where: GE tissue gross energy (KJ/g DM), determined by adiabatic bomb
CP Tissue crude protein content (g/g DM), Kjeldahl analysis
23.6 gross energy content of crude protein (kJ/g DM)[†]
39.6 gross energy content of fat (kJ/g DM)[†]

[†] McDonald *et al.* (1988).

A.1.6.1.3 Ash Content

The tissue ash content was estimated by complete combustion of a known mass (~5 g) of sample at 550 °C for 24 hours.

A.1.6.2 Skin Analysis

In trial 3, the skin was analysed for dry mass, protein content and ash content. The fat content was estimated. Dry mass was determined by freeze drying to constant mass, refer to A1.6.1 above. The skin and hair would not mill due to its high collagen content. Instead, each skin was cut into squares of approximately 0.5 × 0.5 cm and analysis carried out on a representative sample. Due to the relatively large sample pieces, protein was determined using the macro-Kjeldahl technique, the principle of which is the same as that for the micro Kjeldahl technique which is explained in A1.6.1.1 above. A sample mass of 1 g was digested in 20 cm^3 conc H_2SO_4, using 4 g reaction catalyst and anti-bumping granules for 1 h (Tecator Digestion System 20, 1015 Digester). Total nitrogen was then determined by automatic titration (Kjeltec Auto 1030 Analyser). Ash content was determined as described in A1.6.1.3.

As the skin samples were not milled, it was not possible to pellet a representative sample in order to determine the gross energy content using the adiabatic bomb technique described in A1.6.1.2. Although an ether extract method could have been employed to determine the fat content, it was decided that estimation by subtraction from the total dry mass was sufficiently accurate. Fat content was therefore estimated as dry mass minus (total protein + total ash). This calculation was carried out for the carcass and organs data as a check and these results were found to differ from the analysed results by < 2 %.

A.1.6.3 Mammary Analysis

The mammary gland was completely dissected immediately following decapitation, then weighed and stored in a sealed bag at -15 °C pending analysis. Mammary glands from rats in trial 3 were dissected in two halves; the left side had been milked immediately prior to dissection and was discarded. The right side was weighed and stored at -15 °C to await analysis.

Mammary tissue was freeze dried to constant mass (Edwards Pirini 501 freeze dryer). After which, they were individually submersed in liquid nitrogen and ground using a pestle and mortar, freeze dried for a further 24 h and re-sealed in a fresh bag and stored with dessicant at -15 °C.

A.1.6.3.1 DNA Content

DNA content of mammary tissue was quantified using PicoGreen Nucleic Acid Quantitation Reagent (Molecular Probes Europe BV, The Netherlands). An accurately weighed sample (~0.015 g) of ground mammary tissue was homogenised (2 min) in 2 ml TE buffer (0.010 mol Tris-HCl and 0.001 mol EDTA; pH 7.5) using an electric homogeniser with teflon pestle and glass tube (Ciengo LC9), after which the homogenised sample was snap frozen at -80 °C for 24 h, to ensure complete fracture of the nucleus. Calf thymus DNA stock solution was prepared (0.002 g/l TE buffer; pH 7.5) and the exact DNA concentration determined on the basis of absorbance at 260 nm (A_{260}) (Beckman DU-65 Spectrophotometer); an absorbance of 0.04 corresponds to 0.002 g l^{-1} double stranded DNA (dsDNA). The assay was run as follows:

1) PicoGreen Reagent was diluted 1:200 TE buffer (v/v) PH 7.5 and kept in the dark before each assay run. Ten samples were analysed in duplicate, in addition to the standard curve, every assay run.

2) The stock ds DNA solution was diluted to provide a range of samples between 0 and 200 ng dsDNA ml^{-1} TE, 1.0 cm^3 total volume.

3) 5 µl sample was added to 995 µl TE, pH 7.5 and mixed thoroughly.

4) In the dark, 1.0 cm^3 PicoGreen was added to each sample, mixed well and incubated for 5 min at 20 °C.

5) After incubation but still in the dark, the sample fluorescence was measured with an excitation wavelength of 480 nm and emission wavelength of 520 nm on a luminescence spectrometer (Perkin Elmer LS30). To ensure that the sample readings remained in the detection range of the fluorimeter, the instruments gain was set so that the highest concentration dsDNA standard yielded a fluorescence intensity at the fluorimeters maximum (100 %). To minimise the effects of photobleaching, the time for fluorescence measurement was constant for all samples.

6) The fluorescence value of the blank (0 ng dsDNA cm^{-3} TE) was subtracted from that of each of the samples. The corrected data of known dsDNA concentration were used to generate a standard curve. A linear regression equation was calculated and all sample DNA concentrations determined from this. Every assay used a different standard curve and regression equation to ensure accuracy. Sample duplicates differing by more than 5 % were repeated.

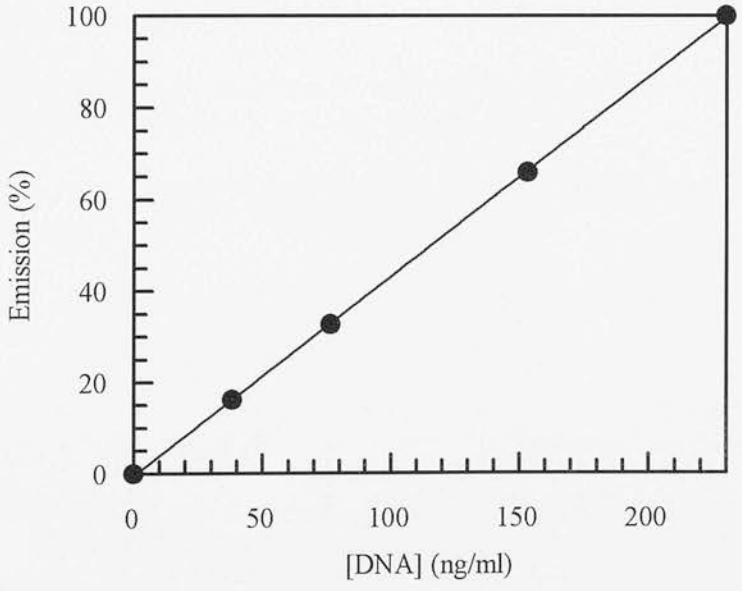

Figure i Typical standard curve of Calf Thymus DNA (dsDNA) in TE buffer, pH 7.5, of which the regression equation (Em (%) = 0.56[dsDNA] - 0.41; r^2=1.00 (P<0.001)) was used to determine the DNA concentration of mammary samples.

A.1.6.3.2 RNA Content

Isolation of high quality RNA is one of the most challenging techniques in modern molecular biology. RNA was extracted from freeze dried, ground tissue using total RNA Isolation Reagent (Advanced Biotechnologies Ltd., Leatherhead).

1) Approximately 0.010 g freeze dried, ground tissue was accurately weighed (4 d.p.) and homogenised with 1 ml of Isolation Reagent using an electric homogeniser with teflon pestle and glass tube (Ciengo LC9).

2) Following homogenisation, homogenate was stored for 5 min at 4 °C to permit complete disslocation of nucleic complexes.

3) Chloroform (0.2 ml) was added to the homogenate and the mixture was shaken vigorously for 15 s and allowed to stand for a further 5 min at 4 °C.

4) The mixture was centrifuged at 12000 × g (4 °C) for 15 min (Heraeus, Megafuge 10R).

5) The upper, aqueous layer containing the RNA was carefully transferred to a fresh tube. The protein and DNA were left in the lower, organic layer and the interphase was discarded.

6) An equal volume of isopropanol was added to the aqueous fraction and the new mixture was stored at 4 °C for 10 min.

7) The mixture was centrifuged at 12000 × g (4 °C) (Heraeus, Megafuge 10R) for 10 min.

8) The RNA formed a white pellet at the bottom of the tube. The supernatent was discarded.

9) The pellet was washed with 1 ml ethanol (75 %) by vortexing and subsequent centrifugation for 5 min at 75000 × g (4 °C) (Heraeus, Megafuge 10R).

10) At the end of the procedure, the pellet was dried under a laminar flow hood, to prevent contamination before being dissolved in 100 µl DEPC treated water by shaking vigourously.

11) A 20 µl aliquot was removed, and made up to 1 ml with DEPC treated water in a fresh tube. This was read at both 260 nm (A_{260}) and 280 nm (A_{280}) (Beckman DU-65 Spectrophotometer). The ratio of A_{260} : A_{280} was always greater than 1.8 indicating that there was little protein contamination. One A_{260} unit was considered to be equal to 40 µg RNA ml^{-1}. This was checked against an RNA stock solution. Tissue RNA content mg/g mammary tissue

was calculated using the appropriate dilution factor and sample weight homogenised.

A.1.6.3.3 Protein Content

A sample of approximately 0.015 g freeze dried, ground mammary tissue was accurately weighed (4 d.p.) and 2 ml NaOH 0.3 mol l^{-1} was added. This was placed in a shaking water bath for 2 h at 55 °C.

Protein content was assayed following the method of Lowry *et al.* (1951).

All samples were repeated in duplicate, and each sample duplicate was also assayed in duplicate.

The following two solutions were prepared immediately prior to the start of each assay run:

① Alkaline copper reagent

Solution A - 50 mg $CuSO_4$ in 10 cm^3 NaK, tartrate (1% w/v)
Solution B - Add solution A to 100 cm^3 Na_2CO_3 (10% w/v) in 0.5 mol l^{-1} NaOH

② Commercial Folin and Ciocalteu's phenol reagent diluted with distilled water (1/10 v/v)

1) 50 µl of homogenate was made up to 1 cm^3 with 0.3 mol l^{-1} NaOH and thoroughly mixed.

2) Standard solutions of bovine serum albumin (BSA) were dissolved in 0.3 mol l^{-1} NaOH at concentrations between 25 - 200 µg cm^{-3} - refer to Figure ii.

3) 1 cm^3 aliquots of both diluted homogenate and standard were added to 20 cm^3 test tubes and kept on ice.

4) 1 cm^3 of alkaline copper reagent (solution ①) was added to all tubes, mixed and allowed to stand on ice for 10 min.

5) 3 cm^3 of Folin reagent (solution ②) was added mixed and the tubes were placed in a shaking water bath at 55 °C for 10 min.

6) After exactly 10 min, samples were cooled to room temperature and absorption at 650 nm (A_{650}) (Beckman DU-62) was measured against a reagent blank.

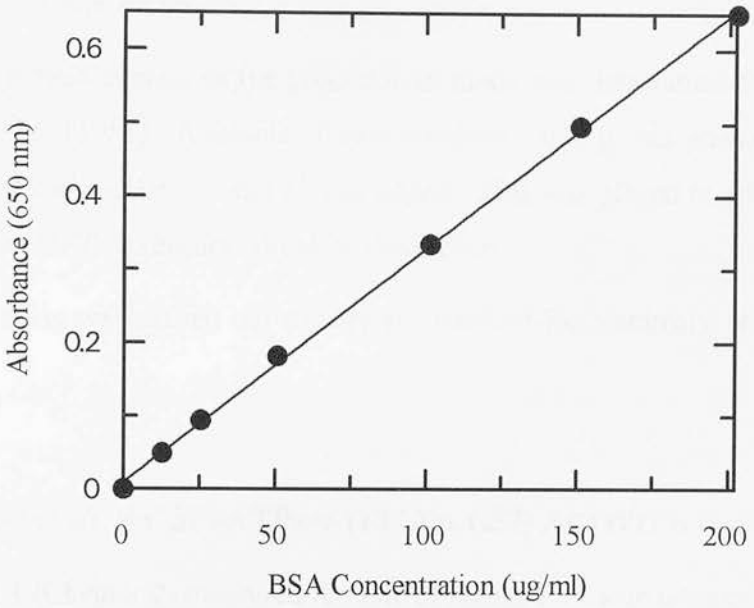

Figure ii Typical standard curve of bovine serum albumin (BSA) in 0.3 mol l^{-1} NaOH, of which the regression equation (Abs_{650} = 0.0103 + 0.0032[BSA]; r^2=0.99 (P<0.001)) was used to determine the protein concentration of samples, following the method of Lowry *et al.* (1951).

The concentration of the standard BSA was plotted against the corresponding absorption. Linear regression analysis was performed and the protein concentration of mammary tissue g g^{-1} calculated from this equation (Figure ii) and the initial sample weight and dilution factor.

A.1.6.4 Liver Analysis

The livers of both the lactating and weanling rats of trial 2 were removed at the time of slaughter, weighed and stored in sealed bags at -15°C for approximately two weeks. They were then freeze dried (Edwards Pirini 501) to a constant mass and ground under liquid nitrogen using a pestle and mortar, then returned to plastic bags and freeze dried for a further 24 h to ensure complete removal of moisture.

A.1.6.4.1 Protein Content

The protein content of the ground liver tissue was determined by the method of Lowry *et al.*, (1951). A sample of approximately 0.015 g was accurately weighed (4 d.p.) and 2 cm^3 NaOH 0.3 mol l^{-1} was added. This was placed in a shaking water bath for 2 h at 55 °C, to ensure complete dissolution.

The assay was carried out exactly as described for mammary protein, section A.1.6.3.3.

A1.7 MAMMARY Na^+,K^+-ATPase (EC 3.6.1.37) ACTIVITY.

Trial 1 (Chapter 2) measured the rate of mammary tissue oxygen consumption and the oxygen consumption attributable to the enzyme Na^+,K^+-ATPase in vitro. Rates of tissue oxygen consumption were measured polarographically in a Rank oxygen electrode (Rank Brothers, Cambridge) which contains a chamber maintained at a constant temperature (37 °C) by pumping preheated water through an outer water jacket. The base of the inner chamber contained two electrodes completely immersed in a solution of saturated potassium chloride and separated from the remainder of the chamber by a thin teflon membrane which is oxygen permeable. The rate of current flow between these two electrodes corresponds to the concentration of dissolved oxygen in the reaction medium and is recorded on a potentiometric chart recorder. As long as the initial oxygen concentration of the reaction medium is known, oxygen concentration following tissue consumption may be calculated.

Before the oxygen electrode can be used, it must first be calibrated.

Electrode calibration:

1) The reaction chamber was rinsed twice with Minimum Essential Medium (MEM)*.

2) Then the reaction chamber was filled with buffer (3 cm^3) at pH 7.4 and 37 °C.

3) Start chart recorder, at a known speed (1 cm min^{-1}) and ensure a steady baseline - represents oxygen concentration of the buffer *.

4) Add sodium dithionite to reaction buffer - to remove all oxygen.

5) Record zero line on chart - difference between two line is the span (cm).

* MEM containing 5 mmol l^{-1} Hepes was air saturated and has a known oxygen concentration (180 nmoles ml^{-1}). Therefore it was possible to calibrate the chart because the span (cm) is equivalent to 540 nmoles O_2 (180 nmoles × 3 cm^3).

Following calibration, two mammary gland slices (~ 20 μm) per rat were obtained by chopping freshly dissected tissue using a tissue chopper (Mickle Lab Engineering Co. Surrey) and were washed in buffer (Minimal Essential Medium (MEM) containing 5 mmol l^{-1} Hepes (pH 7.4; 37 °C), before each slice was placed in a separate reaction chamber with 3 ml of the above buffer. The size of the tissue pieces used was such that the reduction in O_2 content of the incubation medium during the 20-25 min procedure was never greater than 25% of its initial value. Oxygen bubbles were removed and tissue oxygen consumption was recorded on the chart recorder (1 cm min^{-1}) for a period of at least 10 min, this was known as total respiration. After this time, oubain was added to a final concentration of 10^{-4} M and the tissue oxygen consumption recorded for a further 10 min, this was termed Na^+K^+-ATPase independent respiration. The difference between the rate of total respiration rate and that following oubain treatment was termed Na^+K^+-ATPase dependent respiration. Percentage inhibition of the original rate of oxygen consumption can therefore be calculated. The above rates are per snippet, but as snippets were of unequal mass, snippets were standardised to DNA (mg) and the final result was expressed as nmol O_2 mg^{-1} DNA min^{-1}. Samples were standardised to DNA in preference to protein so that tissue respiration per cell could then be obtained, using the equation of Winick and Noble (1965).

DNA content was determined as described in A1.6.3.1.

Calculation:

Span (cm) of O_2 electrode was measured - MEM buffer oxygen concentration before and after dithionite. 540 nmoles O_2 divided by the span $\equiv x$ nmoles O_2 cm^{-1}.

The chart recorder was set at a known speed (V; 1 cm min^{-1})

After the addition of the sample, the chart recorder had both a vertical shift (A cm; oxygen consumtion) and a horizontal shift (T cm; time).

$$\text{Oxygen consumed per min (nmoles min}^{-1}) = \frac{A \times x}{T/V} \text{ nmoles min}^{-1}$$

This calculation is then repeated for the post-oubain period (Na^+K^+-ATPase independent respiration)

Na^+K^+-ATPase dependent = Total respiration - Na^+K^+-ATPase independent

$$\% \text{ Inhibition} = 1 - \frac{Na^+K^+ATP\text{-ase dependent}}{\text{Total Respiration}} \times 100.$$

A1.8 Mammary Lactose Synthetase (EC 2.4.1.22) Activity.

In trials 1 and 3 the lactose synthetase activity of mammary tissue was measured in vitro. Lactose synthetase catalyses the synthesis of lactose from UDP-galactose and glucose and is composed of two sub-units, both of which are required for activity. One of the sub-units, has N-acetyllactosamine (NAL-synthetase) activity on its own, the other sub-unit is identical to the milk protein α-lactalbumin.

$$\text{UDP - galactose} + \text{Glucose} \xrightarrow{\text{Lactose synthetase}} \text{Lactose} + \text{UDP}$$

$$\text{UDP - galactose} + \text{N - acetyglucosamine} \xrightarrow{\text{N-acetyllactosamine synthetase}} \text{N - acetyllactosamine} + \text{UDP}$$

Lactose synthetase activity is proportional to α-lactalbumin concentration as long as the specific activity obtained in the lactose synthetase activity does not exceed three times that of NAL-synthetase (Palmitter, 1969). After that point, α-lactalbumin

becomes limiting. For this reason, NAL-synthetase specific activity has also to be determined; this situation did not occur in these studies.

Method:

1) Mammary tissue (~400 mg) was removed from the right inguinal gland immediately following decapitation.

2) The tissue was chopped (20 μm) using a tissue chopper (Mickle Lab Engineering Co. Surrey).

3) Homogenised in 2 ml of cold (4 °C) 0.02 mol l^{-1} Tris-HCl buffer (pH 7.4) containing: 0.01 mol $MgCl_2$ and 0.001 mol β-mercaptoethanol

using an all glass homogeniser.

The concentration of homogenate ranged from 3-10 mg wet tissue per 50 μml buffer.

4) The final reaction mixture volume contained: 50 μml crude homogenate
40 μml reaction mixture
10 μmm appropriate acceptor

Reaction mixture: 1.33 μmol Tris-HCl (pH 7.4)
0.34 μmol $MnCl_2$
60 nmol UDP-galactose (supplemented with 40000 cpm UDP-[^{14}C]galactose)

5) Three columns per sample were required.
- 2.0 μmol N-acetylglucosamine as an acceptor for NAL-synthetase activity
- 2.0 μmol glucose as an acceptor to measure lactose synthetase activity
- 10 μl distilled water to determine non-specific hydrolysis of UDP-galactose

6) Assays run in duplicate for exactly 30 min in a shaking water bath at 37 °C

Stopped by the addition of 100 μl ice-cold distilled water and cooled on ice

7) Total mixture passed through a column (0.5×4 cm) with a few strands of glass wool to prevent the prewashed (2×500 μl 10^{-3} mol l^{-1} lactose solution) BioRad AG1-X2 anion exchange resin in the chloride form passing stright through the column.

8) Each column eluted three times with 400 μl 10^{-3} mol l^{-1} lactose solution. Total eluate collected directly into Beckmann mini-vials and 2 ml scintillation fluid was added.

9) Samples were counted in a liquid scintillation counter (Beckman LS 5000CE). Specific activities were standardised to DNA content of 50 µl of crude homogenate as described in A1.6.3.1. Specific activity expressed as nmoles lactose formed mg^{-1} DNA / min.

Calculation:

$$\text{UDP - galactose} + \text{Glucose} \xrightarrow{\text{Lactose synthetase}} \text{Lactose} + \text{UDP}$$

Scintillation counting determined the specific activity of lactose (dpm) which had been formed from UDP-[^{14}C]galactose, the stoichiometry is shown in the equation above. From the specific activity of the UDP-[^{14}C] galactose added to each sample it is possible to calculate the molar quantity of lactose formed for each sample over the half hour incubation period.

Specific activity of UDP-[^{14}C] galactose = 3.08 kBq $nmol^{-1}$

$\equiv$ 3080 dps $nmol^{-1}$

3080×60 dpm $nmol^{-1}$ = 184 800 dpm $nmol^{-1}$

$\therefore$ If sample scintillation count = x dpm

$$\text{nmol lactose formed} = \frac{x}{184800} \text{ nmol } 30 \text{ min}^{-1} \text{ sample}^{-1}$$

DNA content of sample was described as in A1.6.3.1 and the results were expressed as nmol lactose mg^{-1} DNA min^{-1}.

A1.9 Specific Liver Histidine Ammonia Lyase (EC 4.3.1.3) Activity.

In trial 2, the specific activity of liver histidine ammonia lyase (EC 4.3.1.3), commonly referred to as histidase, was determined *in vitro* in both adult, lactating and also young, growing rats. Animals were slaughtered by decapitation and a portion (~2 g) was removed from the right, rear lobe of the animal's liver, minced with a

sharp knife and thoroughly homogenated with a glass / teflon electric Potter-Elvehjem homogeniser in 6 ml of buffer at 4 °C.

Buffer: 0.01 mol Tris-HCl (pH 7.4, 4 °C)
0.014 mol $MgCl_2$
0.6 mol Kcl

2) Homogenate was spun for 2 min at 12500×g (Beckman Microfuge)

3) Supernatent was spun for 90 min at 122000×g (MSE Prepsin 65 Ultracentrifuge)

4) Supernatent removed and incubated in a shaking waterbath for 30 min at 55 °C.

5) The supernatent was cooled on ice and the thermally denatured proteins were separated by centrifugation at 66000×g (MSE Prepsin 65 Ultracentrifuge) for 30 min.

6) Reaction initiated by the addition of 30 µl of the second supernatent to 1.5 ml freshly prepared reaction mixture (pH 9.0, 37 °C) in quartz glass cuvettes, stirring thoroughly. Urocanic acid formation was monitored continuously at 277 nm (A_{277}) over a 30 min period at 37 °C using water-jacketted cuvette holders (Beckman DU-65 Spectrophotometer). Samples were incubated in duplicate.

Reaction Mixture: 0.007 mol reduced glutathione
0.01 mol sodium pyrophosphate
0.05 mol L-histidine.

7) Results expressed as mmol urocanic acid mg^{-1} protein/min. Protein content of supernatent determined by the method of Lowry *et al.* (1951).

Calculation:

As it took a few minutes for the reaction to settle down and occasionally the specific activity rate began to plateau towards the end of the 30 min reaction period, results were determined between 5 and 25 minutes of the incubation period.

Molar extinction coefficient (ε) Urocanic acid: = 0.0188 mol l^{-1} cm^{-1}

The slope of the results line was calculted for each sample, (ΔA_{277} min^{-1})

Specific histidase activity (mol l^{-1} min^{-1}) = slope × 0.0188

This was then divided by protein content of 30 µl sample. Protein content was determined by the method of Lowry *et al.* (1951) as described in section A1.6.3.3.

A1.10 Analysis of Milk Composition

Changes in the composition of rat milk in response to dietary protein and energy intake were investigated in trial 3. Milk samples were obtained from rats immediately prior to slaughter on day 1, 5 or 10 of lactation. The left abdominal and inguinal glands of rats were milked only once during lactation after a 2 h seperation from their litter in the morning, at the start of the light period, during this time they had free access to water and, for the animals not on fixed ration regimes, food. This procedure was designed to limit the impact on milk composition of milk stasis (Grigor *et al.*, 1986), serial milking (Keen *et al.*, 1980) and diurnal variations in mammary lipogenesis and lactose synthesis (Williamson *et al.*, 1984; Carrick and Kuhn, 1978). Following this seperation animals were lightly anaethetised (diethyl ether, anaesthetic grade) and injected with 5 international units (i.u.) oxytocin, after which the mammary glands on the animal's left were gently massaged to aid the milk let down process. After 5 min milk samples were obtained by gently hand stripping the teats on the left side and collecting the milk in a 1 ml plastic pipette. The milk was stored in 2 ml eppendorf tubes at -20 °C for approximately 5 weeks, after which it was assayed for lactose, lipid and protein content.

A1.10.1 Milk Lactose

The lactose content of the milk samples was assayed using a lactose test kt supplied by Boehringer Manneheim. Lactose was hydrolysed to D-glucose and D-galactose in the presence of the enzyme β-galactosidase and water. D-galactose was

then oxidised by NAD^+ to galactonic acid in the presence of the enzyme β-galactose dehydrogenase (Gal-DH) as shown in the equations below.

$$\text{Lactose} + H_2O \xrightarrow{\beta\text{-galactosidase}} \text{D - glucose} + \text{D - galactose}$$

$$\text{D - galactose} + NAD^+ \xrightarrow{\text{Gal-DH}} \text{galactonic acid} + NADH + H^+$$

As the amount of NADH produced is stoichiometric with the amount of lactose presence, the increase in NADH is measured using a spectrophotometer (Beckman DU-65 Spectrophotometer) by means of its absorbance at 340 nm (A_{240}).

1) Approximately 20 mg of milk sample was accurately (5 d.p) weighed into an eppendorf.

2) This was deproteinised by the addition of 10 µl of 3 mol l^{-1} trichloroacetic acid (TCA) and 980 µl distilled water, shaken well and left to stand for 10 min.

3) The above mixture was neutralised with 10 µl 0.2 mol l^{-1} NaOH and the protein was pelleted by spinning samples at 1300×g for 30 s (Beckman Microfuge).

4) Supernatent samples (100 µl) and pure (>99.2%) lactose standards were then assayed in duplicate for their lactose content.

The test kit contains 4 reaction solutions used in the assay sequence shown below:

Solution 1) Citrate buffer, pH 6.6; NAD^+; magnesium sulphate and stabilisers
Solution 2) β-galactosidase suspension (59 U/ml)
Solution 3) Potassium diphosphate buffer, pH 8,6; stabilisers
Solution 4) Galactose dehydrogenase (9 U/ml)

	Blank Sample	**Lactose Sample**
Solution 1	0.20 ml	0.20 ml
Suspension 2	0.05 ml	0.05 ml
Sample	-	0.10 ml
Mix and incubate for 15 min at 20 °C		
Solution 3	1.00 ml	1.00 ml
Distilled water	2.00 ml	1.95 ml
Mix and read Absorbance (A_1) at 340nm after 2 min		
Suspension 4	0.05 ml	0.05 ml
Mix and read Absorbance (A_2) at 340nm after 20 min		

For both blank and lactose sample solutions the absorbance difference (ΔA) was calculated ($\Delta A = A_1 - A_2$). The absorbance difference of the blank was then subtracted from that of the lactose solution.

The lactose concentration of the reaction solution was then calculated using the following equation:

$$\text{Lactose (g / d)} = \frac{V \times MW}{\varepsilon \times d \times v \times 1000} \times \Delta A$$

where:
- V final volume (3.35 ml)
- v sample volume (0.10 ml)
- MW molecular weight of lactose
- d light path (1 cm)
- ε absorption coefficient of NADH at 340 nm (6.3)

As x g of milk, where x is the sample weight (mg) was made up to 1 ml this gives mg lactose per ml and therefore divided by the sample weight to obtain mg lactose g^{-1} milk.

Accuracy of this assay, as determined from assaying different concentrations of lactose standards, was 99.4%.

A1.10.2 Milk Protein.

Milk protein content was determined by the method of Lowry *et al.* (1951).

All samples were repeated in duplicate, and each sample duplicate was also assayed in duplicate.

The following two solutions were prepared immediately prior to the start of each assay run.

① Alkaline copper reagent

Solution A - 50 mg $CuSO_4$ in 10 cm^3 Na,K, tartrate (1% w/v)
Solution B - Add solution A to 100 cm^3 Na_2CO_3 (10% w/v) in 0.5 mol l^{-1} NaOH

② Commercial Folin and Ciocalteu's phenol reagent diluted with distilled water (1/10 v/v)

1) Approximately 0.02 g of milk sample was accurately (5 d.p.) weighed into an eppendorf.

2) 2 cm^3 of 0.3 mol l^{-1} NaOH was added, and the solutions thoroughly mixed.

3) This was washed into a 25 cm^3 volumetric flask and made up to volume with 0.3 mol l^{-1} NaOH.

4) Standard solutions of bovine serum albumin (BSA) were dissolved in 0.3 mol l^{-1} NaOH at concentrations between 25 - 200 $\mu g\ cm^{-3}$ - refer to Figure ii.

5) 1 ml aliquots of both the diluted milk samples and the standard solutions were added to 20 cm^3 test tubes kept on ice.

6) 1 cm^3 of alkaline copper reagent (solution ①) was added to all tubes, mixed and allowed to stand on ice for 10 min.

7) 3 cm^3 of Folin reagent (solution ②) was added, mixed and placed in shaking water bath at 55 °C for 10 min.

8) After exactly 10 min, samples were cooled to room temperature and absorption at 650 nm (Beckman DU-62) was measured against a reagent blank.

The concentration of the standard BSA was plotted against the corresponding absorption, refer to Figure ii. Linear regression analysis was performed and the protein concentration of the unknown solutions calculated from this equation (Figure ii) and the initial sample weight and dilution factor.

A1.10.3 Milk Lipid

The total milk lipid content was determined using the extraction procedure developed by Bligh *et al.*(1959).

1) Approximately 50 mg of milk sample was accurately (5d.p.) weighed into an eppendorf.

2) This was washed into a 10 cm^3 centrifuge tube with 2 ml 150 mol l^{-1} NaCl.

3) 4.4 ml chloroform : methanol (1:1 v/v) was added.

This combination ensured that the aqueous, methanol and chloroform volumes are in the ratio 2:2:1.8, which is critical for the procedure to work accurately.

4) Samples were allowed to stand for 10 min

5) Samples centrifuged at 750×g for 5 min (Beckman Mictofuge).

6) The chloroform layer was carefully extracted, and placed in a pre-weighed, dry test tube. The interphase was left so as to avoid distubance of the aqueous layer.

7) A further 2 × 2 cm^3 of chloroform was added to extract the remaining lipid; each time the chloroform layer was removed and added to the pre-weighed, dry test tube.

8) The chloroform was left to evaporate in an oven at 60 °C fo 12 h and the tubes were re-weighed. The diference between this weight, and the initial, dry tube

weight was taken to be the total lipid content of the sample. This was divided by the sample weight and multiplied by 1000 to obtain lipid mg/g.

A1.11 APOPTOSIS / NECROSIS QUANTIFICATION OF MAMMARY TISSUE

A1.11.1 Low Molecular Weight DNA Isolation and Purification

Low molecular weight DNA was isolated from mammary snippets of rats slaughtered in trial 1 by a modified method of Tilly *et al.* (1993a). Immediately after slaughter a snippet of mammary tissue (~0.005 g) from the left inguinal gland was removed in 2 ml Liebevitz buffer, pH 7.4, using a hand held all glass homogeniser. The homogenate was snap frozen at -80 °C for 24 h. Following defrosting, the Liebevitz buffer was discarded and the tissue disrupted by pippetting up and down in 200 μml homogenisation buffer.

Homogenisation buffer:
0.10 mol NaCl
0.01 mol EDTA (pH 8.0)
0.30 mol Tris-HCl (pH 8.0)
0.20 mol sucrose

1) 12.5 µml SDS (w/v) was added and the mixture incubated in a 65 °C waterbath for 1 h.

2) The homogenate was centrifuged at 5000×g for 20 min at 4 °C (Heraeus, Megafuge 10R)

3) The supernatent was removed, and an equal volume of phenol:chloroform:isoamylalcohol (25:24:1 v/v) added. The mixture was shaken for 30 s and spun at 5000×g for 2 min at 4 °C.

4) The upper phase was removed and added to an equal volume of chloroform:isoamylalcohol (24:1 v/v). This was then mixed and spun at 5000×g for 2 min at 4 °C.

5) Upper phase was again removed and an equal volume of ice cold ethanol (100 %) added, the alcohol precipitated the DNA. The alcohol and DNA was frozen at -15 °C for 12 h to maximise DNA precipitation.

6) Following defrosting, the sample was centrifuged at 14000×g for 30 min at 4 °C.

7) The supernatent was carefully removed and the pellet washed in 200 µml of ethanol (80 %).

8) The sample was centrifuged at 14000×g for a further 30 min at 4 °C.

9) The supernatent was removed, and the pellet air dried under a laminar flow hood for 3 h before dissolving in 25 µml sterile distilled water.

10) A 5 µml aliquot was removed and added to 955 µml sterile distilled water. The absorption of this was read at both 260 and 280 nm (Beckman DU-65 Spectrophotometer) and the A_{260} : A_{280} ratio calculated to determine purity. All samples were over 1.85.

11) DNA content of dissolved pellet was calculated ($A_{260}\times 50$ = µg/ml).

A1.11.2 3' End Labelling of DNA and Gel Electrophoresis

1) A solution of 1.5 % (w/v) agarose in TAE buffer, 35 ml total volume, was microwaved until the agarose dissolved. This was poured into a level electrophoresis unit, ensuring displacement of all air bubbles. The comb was inserted.

2) The comb was removed after the gel had set.

3) Each sample was diluted so as to contain 2 µg DNA in 10 µl sterile distilled water, and 24 Bq [α-^{32}P] ddATP (Amersham International); 750 units of terminal transferase; 5 µl 0.010 mol l^{-1} were added before incubating at 37 °C for 60 min.

4) The labelling was terminated by the addition of 5 µl EDTA to each sample.

5) Samples were carefully loaded into 1 cm^3 spin columns and centrifuged for 4 min at 1100×g.

6) Sample volume was measured, and a 1:10 (v/v) dilution of tracking dye was added.

7) Samples were loaded into individual wells on the agarose gel and run at 60 volts until tracking dye had travelled 2/3 the distance of the gel, approximately 105 min.

8) Power was disconnected and gel placed on 4 layers of filter paper and covered with cling film before being vacuum dryed with no heat.

9) The gel was exposed onto X-ray film, for approximately 30 min and developed immediately afterwards. Later, the film was digitally scanned for computer storage.

A1.11.3 Ethidium Bromide Labelling of DNA and Gel Electrophoresis

1) A solution of 1.5 % (w/v) agarose in TAE buffer, 35 cm^3 total volume, was microwaved until agarose dissolved. A couple of drops of ethidium bromide (0.018 μg) were added. This was poured into a level electrophoresis unit, ensuring displacement of all air bubbles. The comb was inserted.

2) The comb was removed once the gel had set, and TAE buffer was poured into the unit so as to just cover the gel.

3) Each sample was diluted so as to contain 10 μg DNA in 20 μl sterile distilled water.

4) A 1:10 (v/v) dilution of tracking dye was added to each sample.

5) Samples (20 μl) were loaded into individual wells on the agarose gel and run at 60 volts until tracking dye had travelled 2/3 the distance of the gel, approximately 3 h.

6) Power was disconnected.

7) Gel was removed and wrapped in cling-film before being examined over a transilluminator. Digital image analysis (Flowgen, IS-500) allowed the image to be saved on computer.

A1.11.4 Histology and Autoradiography

Mammary snippets (~0.10 g) were taken aseptically immediately post-mortem from trial 4 rats and were fixed in Bouin's fixative (70 cm^3 picric acid (saturated solution), 25 cm^3 formaldehyde, 5 cm^3 acetic acid per 100 cm^3) for 24 h. The sample was then dehydrated by replacing the fixative with ethanol (70 % v/v), for a minimum period of 1 h before this was exchanged for fresh ethanol (70 % v/v). After a further 1 h, the 70 % ethanol was replaced with 90 % ethanol for another hour with one change after half an hour. This was replaced with 95 % ethanol for a further 3 hours before being replaced with a number of changes of absolute ethanol. Finally, the ethanol was removed and cedar wood oil was added for a minimum period of 24 h. After this time, the cedar wood oil was replaced with toluene for 30 min, before being replaced with 3 changes of paraffin wax of approximately 2 h each at 60 °C. This was then blocked out in paraffin wax in a plastic mould. Sections of 5-7 μm were cut

using a microtome, the sections were floated out on a heated water bath 55 °C and mounted on gelatin coated slides, and allowed to dry at 20 °C.

The slides were then dipped in K.5 emulsion gel (Kodak, E.U) at 43 °C and drained on a paper towel. The slides were stored on a cold plate for 10 min before storing in the dark for 1 h. Slides exposed in exposure box in a dessicator at 4 °C.

The slides were allowed to come to room temperature before being dipped in developer (Kodak D-19 for 2 min, followed by a 1 min rinse in acetic acid (1 % v/v) and fixed in sodium thiosulphate (30 % w/v) for 4 min. The slides were washed in tap water for 30 min in the dark.

Slides were then stained by dipping in Harris' haematoxylin for 3 min. Following this they were thoroughly washed in running tap water and dipped in acid alcohol (100 cm^3 70 % ethanol and 1 cm^3 conc. HCl). They were quickly rinsed in tap water and dipped in eosin (1:1 working solution) for 15 s. The moisture was removed with 2 dips in 95 % ethanol, each of 5 min followed by 2 dips in 100 % alcohol for 1 min each. The slides were then dipped in xylene 3 times for 10 min each. The slides were finally mounted.

The slides were viewed using a light microscope (Nikon Optihot) for [^{3}H]-thymidine uptake and quantitatively assesed for cells displaying signs of pyknosis. This necrotic index was calculated by counting the number of cells showing uniformly compacted chromatin in 10 alveoli units, every fifth section of the whole explant.

APPENDIX II

DATA FOR ALL EXPERIMENTAL WORK

AND

THE STATISTICAL PROGRAMS

A2.1 INTRODUCTION

The data previously described in the relevant chapter for each rat trial is presented, along with the Genstat5 (ver.3.1.) program used for its analysis. The live animal data (maternal body weight, standardised litter weight and maternal food intakes) are given as the mass recorded at a similar time each morning of the experimental period. In order to compare the effects of dietary treatment on these variables, data were summed over the relevant time period and this cumulated data were analysed by analysis of covariance. The covariate used was maternal body weight on day 1 of gestation, unless otherwise stated.

The Genstat5 (ver.3.1.) programs are presented in a different font to distinguish them from the text. Column identities for the data are described in the pertinent program.

A2.2 DATA FOR TRIAL 1

Maternal Liveweight Data (g)

d - day of lactation

group	d1	d2	d3	d4	d5	d6	d7	d8	d9	d10	d11	d12
Day 1	369.44											
Day 1	380.87											
Day 1	349.11											
Day 1	321.97											
Day 1	369.55											
Day 1	373.71											
H	358.81	357.56	354.45	357.87	352.99	358.23						
H	349.02	345.09	336.16	332.44	338.01	333.07						
H	427.38	410.92	414.65	406.25	409.03	406.24						
H	338.13	342.62	346.04	343.90	340.87	343.78						
H	357.00	353.21	347.74	329.59	325.11	340.85						
H	320.21	322.08	321.17	319.29	325.67	328.63						
H	341.35	346.25	343.94	343.04	340.08	338.34						
L	360.98	344.53	347.10	338.97	330.01	311.98						
L	374.39	365.61	363.75	362.29	342.81	324.34						
L	359.14	349.30	351.26	344.81	343.43	317.63						
L	339.73	337.93	325.28	320.96	310.61	304.58						
L	338.05	329.16	321.95	321.81	314.81	308.53						
L	352.74	353.42	353.61	340.43	328.64	316.85						
LH	372.79	363.56	358.39	360.84	350.90	331.94	355.09	358.76	361.82			
LH	374.41	356.99	353.43	350.85	347.83	344.32	370.00	380.39	390.41			
LH	343.15	344.08	340.57	337.79	333.19	323.49	330.46	336.95	334.84			
LH	330.87	334.67	332.70	326.67	312.99	303.91	313.54	340.50	324.04			
LH	371.63	364.91	365.76	354.31	353.64	346.23	363.48	371.61	378.40			
LH	367.76	367.40	354.71	354.51	348.53	337.61	360.69	365.69	370.39			
LL	382.60	374.24	365.86	355.33	343.90	333.11	319.50	308.80	291.70			
LL	348.79	340.44	337.54	320.25	306.68	295.73	289.02	287.98	283.12			
LL	328.63	325.85	322.27	315.56	308.32	300.42	294.06	291.04	283.62			
LL	338.20	325.42	326.46	319.46	318.87	308.64	298.40	287.45	287.70			
LL	307.35	300.97	300.51	300.15	297.93	291.21	289.54	287.59	278.76			
LL	387.28	386.24	374.47	368.76	365.91	358.72	346.68	341.08	328.36			
LL	377.03	389.21	391.63	369.04	355.50	338.14	324.65	311.66	295.70			
HHH	363.25	342.37	329.41	326.66	329.28	323.12	331.40	334.33	338.65	337.94	334.76	335.19
HHH	345.95	346.96	352.19	331.79	342.60	349.69	340.69	345.53	350.27	352.09	346.51	351.54
HHH	376.55	382.80	388.18	389.35	388.33	387.97	389.05	393.03	392.83	399.00	385.07	399.09
HHH	354.41	344.45	334.90	343.04	341.12	338.29	347.15	346.68	345.88	353.06	360.41	353.34
HHH	407.36	412.26	418.28	411.14	412.39	410.09	405.86	409.95	401.53	399.98	387.91	395.64
HHH	363.75	357.14	359.90	362.67	361.40	358.72	356.06	353.81	359.62	364.55	358.74	343.71
LLL	345.79	336.87	326.23	317.99	305.94	289.55	280.10	272.92	272.15	264.90	265.70	263.07
LLL	381.81	368.85	366.42	356.27	352.65	344.75	334.52	317.82	305.96	302.01	293.21	286.63
LLL	372.00	372.23	370.90	363.64	352.61	344.31	331.91	321.77	309.85	295.01	289.35	285.88
LLL	407.86	394.38	387.24	376.22	363.37	354.14	337.08	324.59	309.84	299.53	293.98	285.84
LLL	357.53	353.05	346.73	341.17	332.19	326.82	315.79	297.60	284.51	273.27	262.39	256.01
LLL	397.73	385.21	375.50	364.55	349.69	332.00	320.00	311.09	293.94	279.40	269.93	260.78
LLL	334.19	337.17	334.27	332.30	325.69	320.38	304.23	293.28	279.11	266.82	254.08	251.29
LLL	398.67	389.49	381.18	376.74	374.59	373.74	366.15	362.03	346.86	361.21	337.04	328.75
LLH	350.16	343.08	336.55	323.34	304.81	292.20	285.19	277.48	276.11	296.98	317.05	323.03
LLH	365.83	352.38	339.07	324.87	315.25	302.66	287.45	280.48	270.64	265.31	282.81	309.02
LLH	378.42	370.95	362.22	355.42	351.62	348.17	336.73	316.83	302.87	333.90	352.10	346.51
LLH	384.66	385.17	375.98	371.99	365.15	349.81	342.12	331.03	317.20	323.99	324.28	317.85
LLH	318.01	311.48	302.58	296.57	282.67	280.84	271.39	268.85	262.41	287.90	305.84	318.46
LLH	395.30	391.02	381.13	365.99	353.27	340.16	326.90	310.56	298.31	315.15	330.62	330.38
LLH	419.43	413.24	400.47	394.63	373.73	366.13	347.33	335.58	326.28	341.27	368.46	356.91
LHH	361.41	357.15	348.94	348.03	335.45	324.86	348.38	352.58	363.15	364.06	369.88	356.83
LHH	372.78	369.08	364.08	359.68	357.99	348.82	348.88	363.13	365.40	355.89	367.10	371.02
LHH	423.68	420.20	410.19	405.21	399.15	378.17	364.16	355.24	335.76	322.86	307.92	296.04
LHH	371.01	368.69	363.82	355.45	341.72	334.87	346.53	361.04	368.74	361.40	356.66	365.32
LHH	390.49	387.67	385.73	382.44	377.04	366.70	352.64	380.73	380.88	370.59	369.07	387.39
LHH	328.95	322.77	318.25	315.11	303.82	290.43	307.53	326.65	337.20	338.87	349.18	347.82

Daily Food Intake (g DM)

d - day of lactation

group	d2	d3	d4	d5	d6	d7	d8	d9	d10	d11	d12
H	15.16	18.22	23.15	25.85	30.90						
H	16.56	22.16	23.59	29.57	28.10						
H	7.41	15.63	18.25	21.96	22.74						
H	18.35	21.05	25.14	29.03	31.10						
H	14.82	16.05	10.63	15.46	33.26						
H	20.83	18.10	25.13	25.97	31.30						
H	17.97	23.22	21.54	28.18	30.16						
L	5.97	12.91	13.32	11.17	4.69						
L	12.00	11.71	18.37	5.43	2.37						
L	16.88	22.34	21.11	20.85	6.35						
L	17.62	17.16	19.31	10.37	10.87						
L	6.58	10.64	16.81	19.38	8.90						
L	18.83	18.59	15.85	12.94	8.14						
LH	13.25	17.86	23.89	17.98	8.91	35.99	32.90	33.59			
LH	12.75	15.38	19.16	22.34	20.18	31.31	35.60	33.77			
LH	16.19	20.13	22.55	23.13	14.39	25.02	27.59	32.03			
LH	19.29	18.44	19.30	12.35	12.05	22.27	31.81	35.80			
LH	12.46	18.96	17.77	23.25	25.75	37.40	38.65	39.51			
LH	16.73	15.11	23.87	20.10	19.88	36.58	34.38	35.11			
LL	22.02	17.00	17.31	16.07	11.83	7.29	16.11	21.82			
LL	10.27	14.99	9.62	3.35	7.17	5.90	12.16	14.47			
LL	12.33	18.37	20.85	17.97	11.14	14.24	15.00	16.91			
LL	12.23	21.91	20.29	27.24	17.25	12.60	12.93	17.83			
LL	18.23	24.08	28.26	23.75	28.09	23.54	26.80	19.91			
LL	18.02	19.27	19.93	19.11	18.56	16.77	16.12	12.59			
LL	22.39	22.42	20.82	7.28	5.92	13.69	10.67	0.35			
HHH	5.99	8.79	11.21	17.17	26.12	25.93	33.14	35.30	31.69	31.73	35.59
HHH	13.16	18.74	9.62	24.67	29.35	27.84	31.44	36.63	36.41	36.66	38.93
HHH	20.56	26.34	26.82	30.60	35.17	35.82	33.64	43.09	45.84	39.37	47.21
HHH	7.44	9.28	21.50	23.30	25.14	33.46	34.21	33.57	37.70	44.66	37.10
HHH	13.28	20.05	20.86	26.15	26.27	29.80	34.99	28.68	31.30	27.87	36.94
HHH	13.26	15.21	25.26	26.96	29.18	27.82	30.65	32.73	40.23	36.90	28.66
LLL	7.25	4.77	7.76	4.67	3.06	5.53	3.32	12.59	10.32	15.76	16.26
LLL	10.88	16.64	16.86	19.25	14.34	14.44	8.90	10.32	13.35	17.59	20.59
LLL	14.47	17.23	18.38	11.73	13.17	6.35	7.20	12.01	6.39	9.95	17.84
LLL	11.42	15.44	14.32	8.45	8.64	8.70	15.96	23.89	12.74	23.83	27.80
LLL	10.43	13.93	18.35	17.56	19.02	12.61	2.26	3.31	5.49	11.49	14.82
LLL	9.16	11.13	5.65	1.29	6.15	0.38	1.59	4.44	5.55	15.08	4.14
LLL	15.08	20.28	21.61	22.54	25.92	10.63	12.92	6.91	10.83	4.82	14.41
LLL	17.01	22.96	22.47	24.51	24.98	24.55	23.78	13.67	38.73	17.93	17.97
LLH	10.11	12.48	8.87	3.77	20.41	14.43	15.00	31.52	27.01	40.62	39.31
LLH	4.54	6.30	2.43	2.09	1.76	0.15	1.56	3.25	5.48	15.90	30.91
LLH	10.96	18.35	17.76	20.74	21.27	12.75	4.83	0.34	25.91	35.11	29.66
LLH	16.91	21.07	23.11	25.84	15.75	13.95	11.68	5.84	21.85	23.33	18.74
LLH	13.04	10.30	14.60	14.19	16.39	11.42	24.53	8.84	32.97	35.20	33.94
LLH	13.76	13.39	14.83	5.54	4.20	3.76	1.21	4.54	22.38	27.93	27.11
LLH	17.47	18.42	20.00	16.16	13.10	3.70	24.37	17.96	44.69	63.59	51.45
LHH	14.23	21.24	22.62	20.45	20.70	32.14	35.50	40.64	39.80	44.13	41.14
LHH	17.54	20.31	21.90	24.89	23.24	19.14	33.96	35.89	32.26	40.46	41.22
LHH	15.47	15.36	20.32	21.26	5.97	6.86	6.22	0.68	3.33	0.84	0.85
LHH	15.70	17.97	16.89	13.06	11.39	28.15	37.55	39.82	35.46	39.77	43.18
LHH	16.92	25.28	25.43	26.00	15.53	20.99	31.81	29.81	33.88	35.58	43.11
LHH	8.66	14.37	16.21	13.12	3.74	28.05	34.67	41.31	38.49	50.19	44.27

Litter Liveweight Data (g)

d - day of lactation

group	d1	d2	d3	d4	d5	d6	d7	d8	d9	d10	d11	d12
H	85.37	95.85	107.39	119.28	137.53	155.08						
H	85.75	101.23	114.70	129.53	146.64	167.29						
H	83.55	93.17	104.96	119.49	139.11	157.31						
H	78.14	89.21	107.91	128.50	149.06	168.97						
H	81.10	92.24	102.37	111.37	125.05	138.25						
H	77.56	92.45	104.24	123.84	144.01	164.77						
H	82.72	93.92	109.68	126.46	148.70	166.94						
L	90.75	93.84	99.54	105.05	110.56	113.48						
L	89.52	97.85	108.75	119.16	126.14	127.20						
L	91.50	105.34	116.66	128.30	136.77	142.43						
L	79.05	93.32	101.38	108.84	115.20	119.61						
L	82.25	87.41	86.43	100.00	107.10	107.98						
L	88.08	99.53	108.88	118.85	121.89	131.11						
LH	87.76	96.64	104.55	114.24	119.90	124.47	137.76	161.95	183.71			
LH	69.01	83.27	94.41	102.09	108.06	113.23	124.13	145.54	162.77			
LH	78.80	85.89	93.96	104.25	113.98	122.71	138.25	159.68	182.09			
LH	73.32	85.18	94.91	103.25	108.41	114.21	128.47	145.95	170.10			
LH	98.71	102.54	110.27	120.37	128.20	133.74	150.66	175.98	210.95			
LH	86.88	97.58	108.32	115.60	123.21	130.60	148.52	175.05	199.76			
LL	80.78	91.70	101.01	113.55	120.40	122.37	125.12	128.22	130.19			
LL	84.56	93.07	99.15	105.12	107.56	114.77	117.26	122.25	127.53			
LL	83.84	93.11	101.38	111.68	121.46	127.40	134.03	139.67	142.83			
LL	75.61	84.58	95.61	107.44	117.81	130.50	136.32	142.57	150.67			
LL	86.46	100.58	111.03	120.79	130.40	141.15	145.74	152.91	160.66			
LL	89.85	99.93	110.34	115.53	122.71	130.01	135.29	142.88	146.57			
LL	96.00	95.41	106.68	117.50	124.17	125.68	130.95	133.57	131.43			
HHH	73.74	82.32	91.31	101.01	112.86	129.18	149.26	165.59	189.84	210.95	229.28	248.11
HHH	78.96	88.46	100.66	115.83	127.86	145.55	164.15	183.84	206.51	233.50	258.61	282.25
HHH	85.52	99.02	118.12	134.53	155.22	179.18	204.08	235.53	267.35	293.85	323.91	343.75
HHH	66.86	74.29	90.47	107.06	124.76	142.97	161.56	186.85	210.26	230.31	260.87	289.74
HHH	87.43	94.02	106.61	123.57	141.06	166.11	184.00	205.72	224.33	245.01	269.29	287.90
HHH	75.57	89.09	102.57	119.58	137.59	159.13	178.77	205.41	229.80	258.90	286.40	307.21
LLL	82.71	89.06	93.64	96.31	101.37	98.80	101.64	104.28	107.89	110.39	117.72	123.08
LLL	86.63	93.94	103.34	114.68	122.59	128.94	134.37	139.05	142.91	144.42	153.17	161.94
LLL	85.78	91.05	97.72	106.21	111.44	118.01	117.64	121.44	124.75	126.63	126.83	131.58
LLL	82.95	96.04	107.65	118.67	125.86	130.42	134.29	134.48	137.46	138.26	137.91	139.20
LLL	88.23	91.42	94.57	105.62	112.42	119.30	121.07	123.60	128.96	129.02	134.11	136.40
LLL	77.17	87.48	97.42	103.42	107.51	109.06	108.92	108.13	109.06	109.04	109.05	109.05
LLL	84.62	87.33	97.22	111.88	118.94	127.78	130.11	133.27	134.24	135.96	136.77	139.19
LLL	70.33	78.33	91.91	104.90	119.61	131.79	143.99	156.60	166.90	181.95	198.50	201.76
LLH	79.65	89.09	92.67	96.51	98.81	103.14	105.52	108.14	112.24	124.01	144.18	171.42
LLH	78.29	82.71	90.39	95.15	99.02	103.08	101.62	102.33	106.30	109.59	116.23	129.79
LLH	76.30	83.59	98.19	105.21	116.06	124.24	130.42	132.73	130.68	145.34	158.97	180.23
LLH	86.47	99.12	111.45	123.11	137.20	142.40	148.08	153.03	151.92	165.46	184.57	196.24
LLH	84.08	93.86	102.37	110.42	113.95	120.00	124.60	130.19	134.66	150.52	172.91	191.82
LLH	83.25	89.36	100.01	104.86	106.78	106.41	108.53	107.79	100.08	117.41	131.70	149.20
LLH	89.53	97.96	114.67	122.64	127.63	131.43	134.12	139.61	143.34	162.35	185.50	210.95
LHH	84.03	98.71	108.21	116.93	129.23	138.63	154.25	177.86	205.44	235.40	257.18	295.07
LHH	76.04	100.59	111.03	121.21	130.16	138.99	153.94	172.52	196.27	223.65	241.68	270.20
LHH	83.68	96.57	109.64	120.50	131.52	139.74	147.31	151.54	151.76	154.20	152.72	147.27
LHH	77.97	91.08	104.42	113.73	121.04	123.89	138.35	163.33	198.26	218.47	247.73	273.74
LHH	87.95	100.75	113.43	126.20	134.75	144.63	152.66	164.65	188.87	213.53	238.41	260.39
LHH	64.19	71.16	80.14	88.36	95.50	98.41	108.60	130.04	152.46	174.88	198.51	228.30

Mammary Composition

Column headings are described in the Genstat5 (ver. 3.1) programs listed below.

Treat	gwt	dm	pc	tp	dnac	tdna	fatc	tfat	RNA	tRNA	R/D
1	318.55	15.41	0.122	1.88	1.20	18.51	0.846	13.03	2.71	41.76	2.26
1	329.24	9.44	0.174	1.64	0.96	9.07	0.809	7.64	4.11	38.75	4.27
1	301.52	7.90	0.181	1.43	1.17	9.22	0.757	5.98	4.56	35.98	3.90
1	293.45	7.08	0.272	1.93	1.45	10.97	0.713	5.05	6.59	46.62	4.25
1	335.88	9.57	0.243	2.33	1.19	11.40	0.778	7.44	12.64	120.92	10.60
1	303.63	15.00	0.132	1.98	0.05	7.13	0.854	12.81	3.11	46.65	6.54
2	340.77	7.31	0.378	2.76	3.58	26.19	0.625	4.57	15.13	110.60	4.22
2	312.07	5.46	0.497	2.71	3.31	18.06	0.495	2.70	22.41	122.36	6.77
2	356.92	9.45	0.306	2.89	1.74	16.44	0.669	6.32	12.32	116.42	7.08
2	344.74	8.37	0.300	2.51	1.79	14.98	0.606	5.07	13.59	113.71	7.59
2	310.61	7.06	0.308	2.17	2.78	19.63	0.662	4.67	13.04	92.06	4.69
2	308.85	7.91	0.273	2.16	3.13	24.72	0.765	6.05	13.92	110.11	4.45
2	296.50	5.92	0.444	2.63	2.24	11.04	0.533	3.15	11.25	66.57	6.03
3	339.58	5.25	0.262	1.38	2.22	11.67	0.670	3.51	8.82	46.28	3.97
3	311.50	6.24	0.268	1.67	1.72	10.75	0.704	4.39	13.51	84.30	7.85
3	324.90	6.90	0.254	1.75	1.17	8.07	0.665	4.59	10.59	73.04	9.06
3	304.94	5.92	0.310	1.84	1.96	11.63	0.623	3.69	13.23	78.32	6.74
3	309.94	5.70	0.420	2.39	1.52	8.69	0.708	4.03	5.08	28.93	3.33
3	327.14	7.39	0.288	2.13	1.51	11.14	0.721	5.33	7.54	55.72	5.00
4	327.83	7.08	0.393	2.78	1.49	10.55	0.596	4.22	13.89	98.31	9.32
4	357.13	7.76	0.355	2.75	3.08	23.91	0.576	4.47	14.73	114.30	4.78
4	329.45	6.28	0.394	2.47	2.10	13.16	0.571	3.58	17.51	109.96	8.36
4	323.94	5.09	0.425	2.16	2.56	13.05	0.500	2.55	17.92	91.19	6.99
4	340.62	7.93	0.353	2.80	2.65	21.05	0.570	4.52	7.57	59.99	2.85
4	324.49	9.25	0.324	3.00	1.69	15.64	0.613	5.67	12.24	113.22	7.24
5	324.04	5.17	0.285	1.47	1.61	8.34	0.648	3.35	8.41	43.48	5.22
5	315.80	4.57	0.378	1.73	1.93	8.84	0.532	2.43	11.21	51.23	5.80
5	313.67	3.53	0.436	1.54	3.28	11.58	0.469	1.66	15.05	53.11	4.59
5	275.96	4.89	0.360	1.76	2.68	13.09	0.623	3.05	12.14	59.34	4.53
5	313.30	5.69	0.342	1.95	1.84	10.47	0.559	3.18	12.45	70.84	6.77
5	331.94	5.93	0.315	1.87	1.65	9.81	0.676	4.01	9.26	54.88	5.60
5	321.88	4.84	0.328	1.59	1.40	6.77	0.592	2.87	7.62	36.88	5.45
6	339.87	6.01	0.401	2.41	2.73	16.41	0.497	2.99	25.83	155.21	9.46
6	317.99	6.63	0.406	2.69	2.16	14.32	0.515	3.41	20.32	134.72	9.41
6	335.18	9.53	0.509	4.85	2.32	22.10	0.465	4.43	17.06	162.58	7.36
6	330.77	7.99	0.436	3.48	2.16	17.22	0.472	3.77	18.60	148.61	8.63
6	362.70	8.45	0.368	3.11	2.61	22.06	0.545	4.60	7.36	62.19	2.82
6	329.71	5.72	0.581	3.32	2.49	14.28	0.659	3.77	9.42	53.88	3.77
7	332.30	3.23	0.431	1.39	3.19	10.30	0.462	1.49	13.39	43.23	4.20
7	329.52	4.69	0.303	1.42	3.70	17.34	0.558	2.62	12.18	57.12	3.29
7	301.45	3.84	0.385	1.48	1.78	6.83	0.574	2.21	7.50	28.80	4.22
7	351.06	2.35	0.491	1.15	5.77	13.55	0.457	1.07	15.08	35.44	2.62
7	309.22	3.99	0.382	1.52	3.26	12.99	0.548	2.19	13.28	52.99	4.08
7	360.56	7.36	0.423	3.11	4.23	15.22	0.512	3.77	5.20	38.27	2.52
7	307.83	2.63	0.453	1.19	4.13	10.85	0.446	1.17	14.23	37.41	3.45
7	343.10	4.55	0.489	2.22	2.47	11.25	0.441	2.01	12.01	54.65	4.86
8	328.79	5.58	0.456	2.54	3.08	17.21	0.525	2.93	12.48	69.64	4.05
8	326.76	4.66	0.372	1.73	2.81	13.11	0.540	2.52	7.37	34.34	2.62
8	343.72	7.56	0.409	3.09	2.76	20.83	0.598	4.52	14.99	113.29	5.44
8	338.81	5.21	0.481	2.51	3.35	17.46	0.484	2.52	17.26	89.90	5.15
8	308.63	6.40	0.390	2.50	2.00	12.77	0.522	3.34	13.98	*	*
8	325.34	6.36	0.482	3.07	2.12	13.49	0.564	3.59	15.58	99.06	7.34
8	343.96	7.25	0.375	2.72	3.00	21.76	0.541	3.93	16.20	117.45	5.40
9	357.67	7.06	0.532	3.76	2.66	18.80	0.430	3.04	19.82	139.89	7.44
9	336.73	6.86	0.421	2.89	2.16	17.93	0.417	2.86	26.32	180.56	10.07
9	356.03	5.56	0.309	1.72	3.52	19.56	0.598	3.32	8.49	47.20	2.41
9	341.15	8.91	0.388	3.46	2.41	21.50	0.530	4.72	19.92	177.44	8.25
9	351.95	6.12	0.433	2.65	4.44	27.20	0.455	2.79	21.39	130.88	4.81
9	333.27	7.36	0.479	3.53	3.04	21.00	0.506	3.72	17.32	127.48	6.07

Mammary Activity

Column headings are described in the Genstat5 (ver. 3.1) programs listed below.

Treat	gwt	tResp	Na/KResp	CelResp	lactsyn	nalsyn
1	318.55	995.82	751.44	244.38	75.84	250.82
1	329.24	543.49	339.89	203.60	47.37	391.04
1	301.52	777.11	670.16	106.96	126.69	529.49
1	293.45	994.34	774.62	219.72	290.07	507.29
1	335.88	589.81	495.02	94.79	70.56	456.71
1	303.63	449.26	351.87	97.39	67.34	110.27
2	340.77	278.94	270.37	8.57	*	*
2	312.07	1114.21	881.46	232.75	185.47	441.57
2	356.92	1117.32	934.03	183.28	*	*
2	344.74	525.10	438.49	86.62	95.12	332.20
2	310.61	640.08	495.64	144.43	123.83	306.72
2	308.85	366.01	324.41	41.60	38.39	109.77
2	296.50	688.65	518.34	170.31	73.13	276.15
3	339.58	938.46	716.18	222.28	*	*
3	311.50	703.69	638.81	64.88	66.89	244.79
3	324.90	475.57	411.93	63.64	*	*
3	304.94	330.66	279.62	51.04	30.55	154.46
3	309.94	1040.57	757.98	282.59	39.98	285.03
3	327.14	*	*	*	65.73	382.93
4	327.83	1975.49	1349.54	625.95	103.79	262.18
4	357.13	1049.66	715.48	334.17	*	*
4	329.45	1934.41	1340.28	594.13	167.68	278.45
4	323.94	626.35	440.14	186.22	138.46	298.25
4	340.62	947.08	617.51	329.57	143.08	256.47
4	324.49	630.24	378.88	251.36	315.20	517.98
5	324.04	448.03	354.25	93.78	38.01	211.58
5	315.80	267.82	207.17	60.66	10.67	27.80
5	313.67	724.39	636.99	87.40	18.46	83.46
5	275.96	785.62	623.86	161.75	35.77	119.44
5	313.30	789.37	593.72	195.65	66.69	376.78
5	331.94	250.28	209.39	40.89	55.19	260.69
5	321.88	652.36	478.44	173.91	47.13	134.43
6	339.87	374.53	351.29	23.24	412.47	563.67
6	317.99	620.71	560.07	60.65	191.76	288.74
6	335.18	601.04	442.66	158.38	403.45	421.52
6	330.77	354.35	276.45	77.90	221.70	251.16
6	362.70	937.83	752.89	184.94	224.78	293.47
6	329.71	535.07	462.37	72.70	92.71	231.20
7	332.30	890.42	671.10	219.32	106.26	308.12
7	329.52	538.73	445.46	93.27	153.25	230.19
7	301.45	410.99	313.38	97.61	24.70	214.16
7	351.06	521.29	440.04	81.25	104.76	425.28
7	309.22	889.95	436.58	453.37	28.58	91.29
7	360.56	279.91	226.28	53.63	62.82	95.41
7	307.83	764.89	551.88	213.01	26.09	126.88
7	343.10	844.87	678.75	166.12	96.13	266.42
8	328.79	687.77	460.83	226.94	145.51	177.92
8	326.76	759.40	649.24	110.16	108.68	156.09
8	343.72	1000.52	650.24	350.28	183.26	221.94
8	338.81	910.02	623.10	286.93	193.39	316.80
8	308.63	*	*	*	216.75	338.48
8	325.34	944.60	711.30	233.30	159.67	142.42
8	343.96	792.53	452.76	339.77	173.26	214.08
9	357.67	1026.71	842.11	184.60	179.05	195.20
9	336.73	741.32	576.09	165.23	284.16	353.98
9	356.03	883.87	676.15	207.72	76.22	85.87
9	341.15	835.20	711.57	123.63	114.84	156.92
9	351.95	781.21	482.17	299.05	127.84	247.76
9	333.27	690.54	563.25	127.29	295.40	437.32

Genstat5 (ver. 3.1) program for the analysis of the data from Trial 1

Live animal - Analysis of Covariance

```
"Analysis of Covariance for Lactational Data - Trial 1 - Oct.'94"
"Trt - Treatment (HHH,LLL,LLH,LHH)
gwt - dam body weight at day 1 gestation (covariate)
damwt1 - dam lactation gain, days 1 to 6
damwt2 - dam lactation gain, days 6 to 9
damwt3 - dam lactation gain, days 9 to 12
damwt4 - dam lactation gain, days 1 to 12
dmi1   - maternal dry matter intake (g) days 1 to 6
dmi2   - intake (g) days 6 to 9
dmi3   - intake (g) days 9 to 12
dmi4   - intake (g) days 1 to 12
litwt1 - litter gain (g), days 1 to 6
litwt2 - litter gain (g), days 6 to 9
litwt3 - litter gain (g), days 9 to 12
litwt4 - litter gain (g), days 1 to 12
"
units [nvalues=27]
factor [levels=4; labels=!t(HHH,LLL,LLH,LHH)]Trt
read [channel=2]Trt, gwt, damwt1,damwt2,damwt3,damwt4,dmi1, \
dmi2,dmi3,dmi4, litwt1,litwt2,litwt3,litwt4

treat Trt
covariate gwt
anova[fprob=yes;print=aovtable,covariates,means]damwt1,damwt2,damwt3,\
damwt4, dmi1,dmi2,dmi3,dmi4, litwt1,litwt3,litwt3,litwt4
```

Mammary Composition and Activity - Analysis of Covariance

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"Analysis of Covariance for Post Lactational Data - Trial 1 - Oct.'94"
"Trt  - Treatment (1,Day1; 2,H; 3,L; 4,LH; 5,LL; 6,HHH; 7,LLL; 8,LLH; 9,LHH)
 gwt  - dam body weight at day 1 gestation
 dm   - mammary dry mass
 pc   - mammary protein concentration (g protein / g tissue)
 tp   - total mammary protein content (g protein)
 dnac - mammary DNA concentration (mg DNA / g tissue)
 tdna - total mammary DNA (mg DNA)
 fatc - mammary fat concentration (g fat / g tissue)
 tfat - total mammary fat (g fat)
 RNA  - mammary RNA concentration (mg RNA / g tissue)
 tRNA - total mammary RNA (mg RNA)
 RD   - RNA : DNA ratio
 tResp- total cellular respiration
 NKResp- Respiration attributable to Na/K Pump
 CelResp- Respiration not atibutable to NA/K Pump
 lactsyn - lactose synthetase activity
 nalsyn - NAL-synthetase activity
"
units [nvalues=59]
factor [levels=9; labels=!t(Day1,H,L,LH,LL,HHH,LLL,LLH,LHH)]Trt
read [channel=2]Trt,gwt,dm,pc,tp,dnac,tdna,fatc,tfat,RNA,tRNA,\
RD,tResp,NKResp,CelResp,lactsyn

treat Trt
covariate gwt
anova [fprob=yes;print=aovtable,covariates,means]dm,cp,tp,dnac,tdna,\
fatc,tfat,RNA,tRNA,RD,tResp,NKResp,CelResp,lactsyn
```

A2.3 DATA FOR TRIAL 2

Maternal Liveweight Data (g)

d - day of lactation

group	d1	d2	d3	d4	d5	d6	d7	d8	d9	d10	d11	d12
MHS	348.89	340.60	337.74	337.15	337.14	337.73	340.41	353.89	338.88	340.57	342.64	342.22
MHS	392.52	379.40	374.19	367.85	364.87	361.38	365.56	368.46	364.00	352.22	349.90	348.42
MHS	380.71	377.39	375.01	372.00	366.90	365.88	371.99	374.75	364.36	368.27	374.22	355.69
MHS	438.46	423.35	408.07	411.08	406.40	402.64	400.57	401.21	400.99	400.36	399.80	402.04
MHS	425.09	420.65	420.62	412.92	413.71	410.43	413.38	406.51	416.00	406.38	401.98	395.49
MHD	377.59	370.54	362.78	362.33	355.69	353.76	357.75	341.47	349.29	341.04	342.45	339.80
MHD	362.98	353.66	360.03	359.67	357.64	362.79	371.34	361.84	367.21	367.05	352.97	351.14
MHD	445.31	425.77	421.13	413.06	417.94	413.74	397.70	401.27	404.30	398.20	400.35	395.49
MHD	445.32	440.43	439.02	439.66	438.17	425.17	403.43	422.12	416.49	417.81	408.23	403.17
MHD	402.88	400.15	397.00	392.40	383.07	375.70	384.34	375.68	384.77	375.62	379.26	365.58
MLS	401.40	384.17	367.04	353.51	337.27	325.54	312.20	302.67	292.78	283.50	278.31	277.11
MLS	312.39	309.51	308.57	303.91	294.10	281.82	264.46	256.40	247.32	252.37	250.76	237.48
MLS	384.83	367.01	359.26	355.29	338.13	322.70	308.36	297.49	286.24	272.95	261.55	254.78
MLS	381.24	366.02	357.36	347.66	332.45	317.15	307.97	303.24	296.94	292.23	286.31	291.57
MLS	301.97	289.96	281.62	278.86	267.14	253.67	244.80	239.18	229.47	231.77	228.02	227.10
MLD	394.66	379.25	376.07	371.18	404.09	371.52	363.54	353.84	347.31	331.10	324.72	315.59
MLD	447.24	440.03	438.30	436.70	421.76	437.97	413.44	415.52	396.00	370.37	346.45	332.58
MLD	382.15	382.27	374.99	369.86	358.48	350.03	338.46	326.28	320.52	307.04	307.04	292.27
MLD	401.36	383.35	368.19	355.88	339.38	321.69	307.59	294.82	282.39	277.36	270.97	283.63

Daily Food Intake (g DM)

d - day of lactation

group	d2	d3	d4	d5	d6	d7	d8	d9	d10	d11	d12
MHS	15.42	21.26	24.11	30.07	33.44	37.32	46.07	34.46	42.45	45.83	44.92
MHS	12.82	14.61	16.59	21.79	22.75	29.28	32.86	30.03	25.47	30.80	35.00
MHS	17.23	20.73	22.26	25.18	29.41	38.74	41.46	38.83	43.56	49.05	41.71
MHS	5.64	9.79	8.63	14.63	23.32	24.90	29.19	31.37	32.14	36.57	38.21
MHS	15.55	19.15	19.89	25.61	26.86	35.39	33.11	39.67	36.20	41.76	39.55
MHD	12.08	13.77	20.51	19.22	23.93	24.91	25.37	31.94	31.16	35.44	37.65
MHD	10.08	21.48	25.25	27.16	33.54	36.54	39.71	41.76	46.81	38.17	43.60
MHD	6.79	13.69	14.65	22.09	24.37	13.45	28.37	37.94	30.85	41.95	37.18
MHD	13.78	18.44	21.18	26.22	22.11	23.12	30.54	29.55	32.40	30.53	34.04
MHD	18.35	18.16	19.44	23.22	25.85	32.32	38.55	34.50	36.38	44.09	40.27
MLS	9.28	2.69	8.65	6.62	8.12	3.38	7.30	6.47	23.60	19.78	30.95
MLS	16.78	21.21	27.25	25.68	11.45	5.33	10.29	13.89	24.16	33.12	23.10
MLS	6.58	9.34	11.65	6.31	4.08	2.02	10.79	7.63	8.29	8.23	4.86
MLS	11.46	13.79	9.05	3.75	2.68	9.16	11.89	24.66	30.41	28.39	39.89
MLS	7.54	7.60	10.36	8.91	5.87	12.57	21.38	35.98	46.42	44.97	54.02
MLD	7.06	15.42	16.33	24.66	29.99	23.12	21.11	22.43	9.22	16.64	22.35
MLD	17.39	21.60	23.54	19.50	37.68	18.51	35.95	18.08	3.21	1.33	0.15
MLD	13.89	16.92	24.15	18.64	20.34	15.20	11.68	14.51	11.71	14.68	15.05
MLD	13.36	19.46	21.74	20.86	30.70	20.52	20.34	20.40	10.18	12.36	9.84

Litter Liveweight Data (g)

d - day of lactation

	d1	d2	d3	d4	d5	d6	d7	d8	d9	d10	d11	d12
MHS	84.58	101.62	117.18	134.62	158.41	182.61	208.03	232.40	261.58	288.58	319.48	347.40
MHS	85.32	100.06	115.57	133.00	152.66	173.62	195.15	218.59	242.23	260.00	283.33	307.72
MHS	77.06	94.26	108.72	124.79	144.88	165.84	190.22	216.37	247.73	273.55	300.85	333.21
MHS	74.27	79.98	88.17	105.21	125.17	141.79	162.65	179.98	203.32	235.59	256.37	281.30
MHS	82.50	96.19	110.96	130.02	151.23	174.53	200.12	227.88	251.58	283.77	312.97	341.36
MHD	82.39	97.01	110.17	128.24	148.40	170.76	189.59	215.94	241.33	270.22	294.06	321.58
MHD	81.78	94.41	107.07	126.22	148.36	173.49	199.52	229.23	257.70	293.76	321.93	349.37
MHD	77.14	84.55	99.17	113.09	129.00	151.24	169.34	186.90	218.67	248.45	278.70	307.38
MHD	87.28	99.85	111.30	126.02	145.36	168.98	185.53	208.88	232.03	257.49	283.25	308.70
MHD	84.73	97.46	109.70	122.63	142.08	160.47	180.68	203.84	231.03	262.10	290.75	322.65
MLS	89.57	93.00	96.92	101.71	105.22	107.60	108.36	113.10	115.97	118.13	123.77	130.92
MLS	79.26	91.68	103.68	115.91	125.47	129.24	133.20	139.87	143.47	147.09	153.69	158.88
MLS	78.13	85.51	94.48	100.90	106.43	107.54	111.57	113.24	114.24	116.48	116.34	116.15
MLS	92.09	101.86	111.69	121.43	124.43	131.48	136.15	137.95	145.88	155.29	162.31	170.06
MLS	72.09	81.17	87.82	94.01	99.27	101.34	106.70	111.00	114.27	119.18	128.90	136.13
MLD	77.90	84.32	95.97	104.24	114.16	125.35	137.78	147.48	157.16	159.34	166.48	175.58
MLD	85.56	92.37	100.77	111.01	126.22	138.56	162.43	175.56	195.51	193.73	191.64	187.55
MLD	77.27	81.05	92.37	102.15	113.76	124.14	131.95	135.88	141.92	147.13	149.55	156.40
MLD	82.89	94.66	101.18	110.46	121.06	127.37	137.44	145.94	154.75	155.25	159.45	162.92

Weanling Liveweight Data (g)

d - day of lactation

	d1	d2	d3	d4	d5	d6	d7	d8	d9	d10	d11	d12
VLD	146.70	142.54	141.81	138.40	140.20	139.12	139.13	138.85	142.31	144.13	142.92	146.25
VLD	154.75	148.93	145.78	144.14	140.59	141.90	142.12	140.18	143.41	144.28	145.30	148.91
VLD	156.06	151.14	150.05	150.07	147.09	148.44	148.48	145.67	146.90	148.61	151.25	151.24
VLD	153.14	148.76	145.64	147.26	141.17	141.58	144.13	141.08	144.17	141.98	144.13	142.47
VLD	148.06	141.45	143.10	141.89	141.37	139.45	142.61	143.42	144.57	143.25	143.30	142.30
VLD	140.23	132.21	130.91	129.53	128.19	129.83	131.83	131.05	133.09	134.52	136.25	135.24
LD	139.45	137.42	139.94	140.31	139.29	144.55	148.58	149.94	154.62	152.63	159.37	164.98
LD	128.45	125.96	129.59	133.10	135.99	138.74	142.56	145.92	149.88	147.41	153.64	158.41
LD	138.57	135.69	135.38	136.12	139.32	140.39	143.32	141.93	147.31	151.49	155.93	158.71
LD	148.51	149.29	153.49	153.22	153.78	158.52	163.60	167.26	167.33	168.79	175.70	181.57
LD	143.33	139.05	134.27	140.16	147.74	151.97	152.47	159.13	164.52	169.17	169.47	169.61
LD	145.78	148.20	148.69	147.68	148.66	155.22	161.33	161.89	161.70	169.23	175.75	176.25
HD	149.22	151.09	155.44	156.98	160.96	162.75	167.71	173.63	177.22	178.15	180.45	186.37
HD	140.73	143.09	146.91	150.91	151.68	157.21	161.46	165.88	168.29	172.42	176.88	182.40
HD	145.95	148.74	153.72	156.58	158.34	164.31	168.71	174.99	177.05	179.76	184.83	189.74
HD	144.04	147.45	152.94	156.00	160.56	162.40	168.94	173.93	176.54	183.59	189.39	194.52
HD	144.81	148.04	152.36	157.04	159.65	161.86	167.05	173.76	177.40	180.03	184.08	189.92
HD	135.52	137.17	139.00	143.66	145.72	148.72	151.56	156.54	160.90	165.27	169.07	171.75
VLS	144.37	142.90	140.07	138.18	136.16	137.19	138.47	137.36	138.03	140.88	144.30	144.57
VLS	146.21	144.28	142.28	139.45	139.94	141.38	141.07	139.50	140.74	139.72	141.85	142.28
VLS	137.27	136.31	135.75	131.80	128.71	129.30	129.97	128.31	131.38	130.74	132.20	129.11
VLS	155.23	152.12	146.57	140.94	144.56	143.39	148.59	144.02	146.50	148.34	146.96	147.92
VLS	151.65	150.53	145.51	149.13	146.63	145.87	145.51	145.59	147.10	145.00	147.03	149.96
VLS	150.60	150.12	144.67	145.00	144.94	142.92	142.27	139.97	143.67	149.96	146.60	149.41
LS	151.60	152.08	150.70	157.95	162.62	166.16	166.16	174.55	177.96	183.09	182.14	190.72
LS	151.59	152.15	156.38	158.21	160.10	159.04	164.92	166.00	165.70	163.57	170.14	173.27
LS	142.00	141.61	144.48	150.91	156.10	162.39	165.80	166.94	174.08	178.60	182.26	181.21
LS	145.21	145.59	146.91	151.17	154.71	158.59	160.06	164.50	167.84	172.23	175.02	173.30
LS	146.87	147.32	148.95	148.96	156.60	159.97	161.57	161.77	161.29	165.27	168.84	170.85
LS	149.10	150.67	150.81	154.26	157.60	157.18	163.61	166.90	170.99	169.28	176.29	180.89
HS	145.60	147.28	150.41	153.66	150.53	157.38	164.35	169.36	172.79	178.91	184.52	189.28
HS	144.46	147.32	153.75	155.29	160.09	167.69	169.74	169.90	170.11	178.39	181.00	183.56
HS	135.48	139.37	144.83	146.55	149.82	153.27	159.10	166.25	162.85	170.31	174.12	180.92
HS	147.96	151.07	151.23	156.27	160.42	165.56	163.86	168.09	172.79	176.55	175.56	181.86
HS	143.79	149.09	152.80	153.38	157.61	161.46	164.43	165.97	167.97	171.75	175.11	180.60
HS	139.91	143.11	146.64	151.69	154.64	156.62	162.18	166.03	170.88	172.39	175.66	181.44

Weanling Food Intake Data (g DM)

d - day of lactation

	d2	d3	d4	d5	d6	d7	d8	d9	d10	d11	d12
VLD	5.19	8.83	7.77	8.46	8.16	10.17	10.26	11.72	10.12	11.14	11.54
VLD	3.95	8.24	6.65	7.87	8.80	8.59	6.79	12.11	11.48	10.65	13.35
VLD	6.32	6.67	8.07	8.84	9.91	9.53	8.02	11.30	13.39	11.46	11.58
VLD	4.28	10.51	9.78	5.10	8.31	10.66	7.84	11.14	9.41	12.60	7.52
VLD	5.93	11.50	9.18	10.17	9.65	13.20	12.76	12.23	10.71	7.44	9.82
VLD	2.36	4.51	9.45	10.72	11.63	12.59	11.49	13.71	11.94	11.56	10.68
LD	6.19	11.43	10.31	9.92	12.59	12.09	11.23	10.94	11.22	13.91	12.97
LD	8.60	11.45	11.34	12.37	12.11	10.92	11.24	12.45	9.63	12.65	13.65
LD	7.44	9.25	9.20	11.90	10.22	11.20	10.10	12.64	12.73	13.35	11.19
LD	12.54	11.50	10.45	12.10	10.08	14.03	12.50	12.33	12.74	16.54	14.90
LD	3.97	3.57	12.56	18.51	14.10	12.67	14.08	14.36	15.92	11.74	10.93
LD	11.23	10.35	13.05	12.55	17.00	13.11	10.38	10.70	14.18	15.84	12.97
HD	11.61	12.63	11.50	13.47	12.70	13.04	15.07	13.16	13.32	13.35	14.65
HD	10.98	11.22	12.90	12.43	13.81	11.79	17.27	11.35	18.31	12.63	14.03
HD	12.10	10.75	11.09	11.11	12.67	13.12	13.77	11.99	13.57	14.37	16.60
HD	11.26	11.29	10.71	12.94	11.24	14.43	14.54	10.83	15.56	15.73	15.79
HD	10.26	11.32	11.50	11.74	11.65	11.77	14.03	11.77	13.26	14.08	14.60
HD	9.60	10.08	11.73	11.11	11.05	10.94	11.14	11.94	12.82	11.79	11.34
VLS	7.28	6.22	6.79	9.33	10.34	12.16	9.43	10.80	14.46	13.37	10.63
VLS	6.54	7.57	7.58	11.88	9.99	13.83	16.13	18.88	12.85	24.35	23.32
VLS	5.41	7.37	6.86	7.43	8.21	8.47	10.76	11.62	8.84	9.93	7.08
VLS	6.70	6.56	5.54	10.21	10.15	15.91	23.80	23.76	27.39	10.81	24.52
VLS	9.95	8.20	13.97	11.34	8.69	9.88	14.60	12.09	8.24	12.91	15.42
VLS	9.62	7.21	8.98	9.45	11.11	9.40	9.78	25.34	29.75	0.25	26.23
LS	11.86	10.05	16.87	14.39	14.87	12.43	16.29	14.73	16.25	11.69	15.72
LS	11.56	10.47	12.54	14.09	9.80	13.25	13.28	10.00	8.53	10.94	11.89
LS	9.48	12.70	15.11	15.04	15.82	14.29	10.49	16.29	15.42	14.75	10.30
LS	11.57	9.51	13.87	12.75	14.05	9.47	14.16	13.60	13.95	11.39	11.00
LS	10.41	13.75	11.93	15.88	14.12	13.02	10.97	10.72	11.80	12.40	11.44
LS	9.87	10.24	12.54	13.19	10.24	12.37	15.08	13.53	9.31	13.15	13.90
HS	14.08	12.03	11.77	9.85	13.06	13.88	14.20	13.60	15.48	15.59	14.44
HS	14.37	12.17	11.81	11.61	16.61	9.14	10.59	11.81	18.82	13.18	13.09
HS	11.31	11.34	12.70	13.06	12.32	17.87	11.62	11.86	16.62	16.85	20.27
HS	11.02	10.94	11.84	11.72	10.45	10.55	11.34	11.04	12.03	10.40	11.87
HS	11.22	11.39	11.80	11.36	14.13	13.18	10.83	12.37	11.90	13.74	11.98
HS	10.41	11.38	10.95	11.37	10.46	11.89	11.98	13.55	10.98	12.01	14.75

Liver Data

Lactating Animals

Trt	bwt	DM	Uro	Ash	cPro	tPro
1	348.89	4.06	5.7841	47.31	729.60	2.96
1	392.52	4.69	2.4245	40.86	613.84	2.88
1	380.71	6.47	2.1597	31.83	479.98	3.10
1	438.46	5.34	2.3138	38.18	583.66	3.11
1	425.09	6.95	2.0722	41.71	610.04	4.24
2	377.59	4.96	2.5892	35.91	527.22	2.62
2	362.98	4.49	5.4781	43.17	644.06	2.89
2	445.31	4.33	2.0250	42.78	641.10	2.78
2	445.32	7.11	1.0934	26.76	410.88	2.92
2	402.88	4.37	2.3097	42.31	603.41	2.64
3	401.40	3.06	1.1799	44.70	581.51	1.78
3	312.39	1.32	2.1210	46.57	616.60	0.81
3	384.83	2.66	1.2362	39.54	505.09	1.34
3	381.24	4.31	1.4982	34.47	471.83	2.03
3	301.97	2.46	1.2868	46.02	629.78	1.55
4	394.66	3.28	0.9901	39.24	595.51	1.96
4	447.24	3.43	2.1150	35.79	507.48	1.74
4	382.15	2.79	1.1139	33.53	636.40	1.78
4	401.36	6.09	1.9433	25.55	319.07	1.37

Weanling Animals

Trt	bwt	DM	Uro	Ash	cPro	tPro
1	146.70	2.24	0.4113	27.18	462.58	1.04
1	154.75	2.28	0.6354	37.28	466.28	1.06
1	156.06	1.75	1.2921	47.52	671.70	1.18
1	153.14	1.63	0.9829	45.20	645.32	1.05
1	148.06	1.75	0.5630	43.72	613.31	1.07
1	140.23	1.92	1.0494	48.79	571.88	1.10
2	139.45	2.21	5.1321	44.27	573.73	1.27
2	128.45	2.23	13.0820	45.17	658.53	1.47
2	138.57	2.18	2.1521	42.13	628.45	1.37
2	148.51	2.33	4.9625	45.57	680.46	1.59
2	143.33	2.29	0.7489	44.04	610.97	1.40
2	145.78	2.52	1.2440	41.73	623.51	1.57
3	149.22	2.99	9.2407	39.47	536.84	1.61
3	140.73	3.26	11.1993	39.14	551.25	1.79
3	145.95	3.22	5.3560	41.10	600.50	1.93
3	144.04	2.96	7.3754	45.17	654.76	1.94
3	144.81	2.76	1.0826	33.34	596.35	1.64
3	135.52	2.69	1.7379	41.61	606.44	1.63
3	144.37	2.14	14.1959	39.89	484.07	1.04
4	146.21	1.69	1.5818	17.93	585.21	0.99
4	137.27	1.54	1.1747	45.21	596.50	0.92
4	155.23	2.03	0.6446	40.45	548.45	1.11
4	151.65	1.89	0.8728	38.57	565.18	1.07
4	150.60	1.89	0.5833	40.28	550.31	1.04
5	151.60	2.47	4.1422	42.13	619.61	1.53
5	151.59	2.20	4.1343	42.87	602.97	1.33
5	142.00	2.18	4.8440	46.91	659.31	1.44
5	145.21	2.22	5.8198	46.38	616.41	1.37
5	146.87	2.25	0.8625	41.54	569.78	1.28
5	149.10	2.32	0.6627	44.40	641.92	1.49
6	145.60	2.92	1.6856	40.35	568.40	1.66
6	144.46	2.64	6.1506	33.55	579.25	1.53
6	135.48	3.02	0.7012	43.04	583.89	1.76
6	147.96	2.93	0.6522	38.57	554.32	1.63
6	143.79	2.91	1.2250	39.36	516.79	1.50
6	139.91	2.78	1.3753	37.74	565.52	1.57

Genstat5 (ver.3.1) Programs for Trial 2

Live Animal Data (Lactating Animals) - Analysis of Covariance

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"Trial 2 - Oct.'94 (April 1996)"
"Trt - Treatment
 bwt - Day 1 gestation Weight
Wtg1 - Dam weight change (g)  days 1-2
Wtg2 - Dam weight change (g)  days 1-3
Wtg3 -                        (g)    days 1-4
Wtg4
Wtg5
Wtg6
 .
 .
 .
 DMI1 - Cumulative Dry Matter Intake (g)     days 1-2
 .
 .
 .
P1 - Cummulative Pup Weights (g)     days 1-2
 .
 .
 .
"
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output [width=80] 1
units [nvalues=19]
factor [levels=4; labels=!t(FHS, FHD, FLS, FLD)]Trt

read [channel=2]Trt, lwt, gwt, Wtg1, Wtg2, Wtg3, Wtg4, Wtg5, Wtg6, \
Wtg7, Wtg8, Wtg9, Wtg10, Wtg11, DMI1, DMI2, DMI3, DMI4, DMI5, DMI6, \
DMI7, DMI8, DMI9, DMI10, DMI11, P1, P2, P3, P4, P5, P6, P7, P8, P9, \
P10, P11

treat Trt

"Analysis of covariance using day 1 gestation weight as covariate"
covariate gwt
anova [fprob=yes; print=aovtable, covariates, means]Wtg1, Wtg2, \
Wtg3, Wtg4, Wtg5, Wtg6, Wtg7, Wtg8, Wtg9, Wtg10, Wtg11, DMI1, DMI2, DMI3, \
DMI4, DMI5, DMI6, DMI7, DMI8, DMI9, DMI10, DMI11, P1, P2, P3, P4, P5, P6, \
P7, P8, P9, P10, P11
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Live Animal Data (Weanling Animals) - Analysis of Covariance

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"Trial 2 - Oct.'94 (April 1996)"
"Trt - Treatment
 bwt - Day 1 Treatment Body Weight (Covariate)
 Wtg1 - weanling weight change days 1-2
 Wtg2 - weanling weight change days 1-3
 Wtg3 -                         days 1-4
 Wtg4
 Wtg5
 Wtg6
  .
  .
  .
  .
 DMI1 - Cumulative Dry Matter Intake (g)
  .
  .
  .
"
close channel=2
open 'cumdatw.gdt'; channel=2; width=300
units [nvalues=36]
factor [levels=6; labels=!t(WVD, WLD, WHD, WVS, WLS, WHS)]Trt
read [channel=2]Trt, bwt, Wtg1, Wtg2, Wtg3, Wtg4, Wtg5, Wtg6, \
Wtg7, Wtg8, Wtg9, Wtg10, Wtg11, DMI1, DMI2, DMI3, DMI4, DMI5, DMI6, \
DMI7, DMI8, DMI9, DMI10, DMI11
treat Trt

"Analysis of covariance using day 1 treatment weight as covariate"
covariate bwt
anova [fprob=yes; print=aovtable, covariates, means]Wtg1, Wtg2, \
Wtg3, Wtg4, Wtg5, Wtg6, Wtg7, Wtg8, Wtg9, Wtg10, Wtg11, DMI1, DMI2, DMI3, \
DMI4, DMI5, DMI6, DMI7, DMI8, DMI9, DMI10, DMI11
```

Liver Data (Lactating Animals) - Analysis of Variance

```
"Trial 2 - Oct.'94 (January 1996)"
"Trt - Treatment
 bwt - day 1 lactation weight
 DM  - liver dry matter (g)
 Uro - Urocanic acid formed (mmol / min / mg Protein
 Ash - Liver ash content (g/kg)
 cPr  - liver protein content (g/kg)
 tPr - Total liver protein (g)
"
units [nvalues=19]
factor [levels=4; labels=!t(FHS, FHD, FLS, FLD)]Trt
read [channel=2]Trt, bwt, DM, Uro, Ash, cPr, tPr

"Analysis of covariance using day 1 lactation weight as covariates"
treat Trt
covariates bwt
anova [fprobabilities=yes; print=aovtable, covariates, means]DM, Uro,Ash, cPr, tPr
```

Liver Data (Weanling Animals) - Analysis of Covariance

```
"Trial 2 - Oct.'94 (January 1996)"
"Trt - Treatment
 bwt - day 1 lactation weight (Covariate)
 DM  - liver dry matter (g)
 Uro - Urocanic acid formed (mmol / min / mg Protein)
 Ash - Liver ash content (g/kg)
 cPr  - liver protein content (g/kg)
 tPr - Total liver protein (g)
"
units [nvalues=19]
factor [levels=4; labels=!t(FHS, FHD, FLS, FLD)]Trt
read [channel=2]Trt, bwt, DM, Uro, Ash, cPr, tPr

"Analysis of covariance using day 1 lactation weight as covariates"
treat Trt
covariates bwt
anova [fprob=yes; print=aovtable, covariates, means]DM, Uro,Ash, cPr, tPr
```

A2.4 DATA FOR TRIAL 3

Maternal Liveweight Data (g)

	d1	d2	d3	d4	d5	d6	d7	d8	d9	d10
Day 1	349.90									
Day 1	443.89									
Day 1	335.17									
Day 1	403.21									
Day 1	369.94									
Day 1	427.28									
Day 5	365.19	348.12	341.05	339.52	333.11					
Day 5	410.61	393.84	380.34	375.25	364.32					
Day 5	425.16	429.46	424.06	420.90	422.57					
Day 5	361.05	351.50	344.15	338.75	339.82					
Day 5	356.24	355.73	347.87	339.60	338.83					
Day 5	404.17	377.12	365.43	359.30	340.63					
i	400.85	395.93	382.97	367.30	350.52	338.42	323.81	311.93	299.99	291.97
i	364.58	353.45	358.98	356.12	355.18	334.02	315.74	302.56	288.23	268.85
i	348.61	332.97	324.72	307.91	298.47	279.05	267.81	271.21	262.54	262.66
i	362.09	344.15	340.88	329.14	319.90	302.85	291.50	282.28	273.64	269.54
i	390.50	382.16	383.59	367.44	358.50	343.72	328.80	320.96	304.45	287.62
i	337.05	329.46	323.57	319.43	312.95	299.38	281.39	281.70	267.89	255.17
ii	325.16	308.70	298.85	289.23	280.96	270.58	269.11	258.92	250.32	243.25
ii	348.23	342.94	340.23	338.21	321.80	305.11	293.09	291.36	288.39	265.20
ii	413.66	409.03	402.05	392.97	388.26	364.53	357.35	342.59	335.62	323.53
ii	348.05	337.56	336.85	328.47	325.99	311.87	297.23	289.88	282.02	272.52
ii	369.00	361.23	356.40	349.85	330.66	316.93	305.93	301.30	294.32	286.59
ii	371.75	363.31	345.93	343.11	332.26	319.82	302.50	292.14	280.47	269.04
ii	390.75	382.83	379.43	379.49	372.40	365.76	343.69	329.41	318.99	299.38
iii	350.36	342.61	337.50	327.26	319.98	306.34	297.52	290.64	281.28	272.69
iii	335.97	334.74	326.77	327.11	323.82	315.33	303.19	294.16	280.59	271.30
iii	373.73	371.83	358.97	356.41	358.46	342.47	331.32	319.37	309.11	303.96
iii	349.81	330.93	315.61	296.67	286.18	286.70	284.13	273.70	265.77	259.79
iii	362.83	355.28	356.31	347.93	340.86	333.81	320.85	310.76	298.21	291.68
iii	384.45	383.17	377.76	368.03	364.09	347.17	340.03	329.10	324.95	313.46
iv	378.12	360.33	352.25	353.35	343.32	335.28	328.38	320.56	313.21	303.49
iv	391.14	375.58	370.66	352.97	335.64	332.74	336.77	325.71	317.71	310.96
iv	347.93	339.60	332.80	322.18	304.57	294.26	295.12	286.86	285.28	277.42
iv	393.95	386.26	389.01	380.29	371.82	363.89	350.99	345.27	337.22	328.17
iv	347.62	330.04	326.81	325.38	312.55	307.84	306.47	299.69	291.62	287.71
iv	360.04	344.58	335.94	323.04	310.80	311.15	301.14	304.69	300.55	291.40
v	376.91	367.57	361.46	354.70	337.84	333.82	327.83	319.92	312.19	307.04
v	353.13	336.80	333.51	320.61	309.54	308.84	309.98	299.95	293.37	287.97
v	385.25	381.35	279.29	376.37	370.36	358.29	354.21	347.97	337.51	331.02
v	339.39	320.25	306.28	293.22	280.95	292.66	294.84	287.28	284.32	278.31
v	370.54	357.63	337.87	322.88	305.77	320.22	311.79	307.94	292.92	291.70
v	357.02	334.18	336.56	334.57	325.98	325.66	325.35	318.41	312.55	306.03
vi	362.44	345.36	348.35	340.55	321.97	326.50	325.76	334.01	321.59	314.79
vi	352.60	343.90	335.77	334.59	320.83	322.79	320.93	313.48	313.62	307.49
vi	325.19	315.77	318.42	310.03	309.07	314.44	310.78	306.40	296.87	301.52
vi	319.10	308.15	308.30	299.29	293.76	298.48	304.55	299.95	293.71	287.93
vi	390.37	384.22	375.29	365.77	360.27	363.01	359.61	356.78	347.90	341.58
vi	360.96	354.50	349.88	341.85	334.71	331.85	328.63	324.91	316.84	316.61
vii	369.79	348.89	334.95	320.54	304.79	317.97	328.67	320.85	315.71	311.28
vii	366.33	346.55	343.58	328.52	315.01	329.23	322.19	333.63	332.38	329.41
vii	335.93	321.54	317.48	316.37	303.78	314.79	318.61	316.64	310.16	304.17
vii	410.65	391.07	383.45	364.89	350.52	363.11	363.68	371.21	365.68	361.56

vii	353.74	344.95	332.74	324.36	328.63	327.65	328.89	333.62	330.15	323.20
vii	334.57	321.99	307.05	291.96	278.27	295.24	304.63	296.59	293.86	285.99
viii	392.71	384.73	379.95	373.21	363.81	361.14	357.87	354.65	347.46	338.61
viii	380.77	370.61	366.58	361.24	353.49	352.80	342.86	341.23	333.57	327.52
viii	324.14	314.61	311.65	309.56	291.33	295.21	297.18	288.91	282.94	280.50
viii	390.49	395.59	387.63	389.76	381.66	368.96	365.64	362.18	355.11	343.78
viii	304.94	303.13	301.94	292.74	292.34	286.56	277.30	275.23	269.47	264.58
viii	355.03	339.26	328.33	339.40	325.12	324.53	312.40	312.84	307.25	295.56
ix	385.06	370.22	352.85	338.74	322.15	338.70	344.68	339.11	343.03	340.61
ix	363.35	358.28	360.20	352.00	344.62	339.58	345.76	363.07	353.46	347.12
ix	378.74	357.37	353.07	339.13	321.10	335.73	345.96	353.77	355.48	346.57
ix	350.45	339.35	334.95	326.95	321.13	330.68	339.97	338.27	331.71	329.28
ix	358.91	355.27	329.74	318.01	305.57	319.38	339.50	340.89	352.88	358.28
ix	342.78	341.66	345.07	335.62	326.11	336.03	335.30	351.98	346.63	343.97
H	361.13	355.01	354.29	344.24	334.76	341.02	335.74	337.64	342.18	335.49
H	339.74	332.79	328.74	319.87	325.87	327.23	326.48	322.80	333.22	329.82
H	420.11	411.76	407.03	410.25	410.68	410.83	414.28	407.72	411.67	408.15
H	346.48	336.61	340.31	340.30	344.87	350.03	341.60	343.93	339.60	357.10

Maternal Food Intake Data (g DM)

	d2	d3	d4	d5	d6	d7	d8	d9	d10
Day 5	9.55	15.50	21.40	17.97					
Day 5	6.71	5.48	10.01	9.14					
Day 5	19.48	18.77	26.12	29.03					
Day 5	14.38	15.15	19.48	18.86					
Day 5	11.24	15.68	20.69	19.74					
Day 5	3.32	20.73	13.50	5.64					
i	13.66	10.59	6.42	8.22					
i	14.26	24.81	27.58	27.36					
i	10.17	13.79	5.99	13.27					
i	7.57	14.26	16.81	15.69					
i	14.12	25.93	22.49	18.89					
i	19.19	15.08	21.03	18.93					
ii	6.70	10.50	11.87	13.95					
ii	16.50	23.24	26.84	16.91					
ii	16.54	13.13	18.22	13.95					
ii	8.97	18.42	17.29	18.97					
ii	13.15	13.51	21.34	7.38					
ii	12.87	10.70	19.16	11.87					
ii	18.48	18.56	24.80	20.53					
iii	18.71	22.62	20.44	21.59					
iii	17.79	20.95	27.67	6.94					
iii	22.75	17.96	25.02	29.50					
iii	4.36	1.53	0.85	0.98					
iii	13.80	20.32	18.63	22.31					
iii	16.86	17.32	22.78	19.85					
iv	5.51	15.29	23.42	15.12					
iv	6.74	9.76	8.11	4.37					
iv	5.89	9.62	8.50	4.82					
iv	7.77	14.41	26.90	24.86					
iv	6.17	7.38	22.08	15.90					
iv	4.17	8.22	4.92	4.47					
v	16.53	18.41	21.11	12.70					
v	10.03	9.45	7.46	8.66					
v	20.14	21.57	25.35	24.80					
v	3.02	1.17	1.49	0.97					
v	4.40	3.71	3.30	0.77					
v	3.93	18.09	21.98	16.67					
vi	4.57	15.28	21.40	17.97					
vi	17.63	17.59	21.70	13.86					
vi	4.07	17.57	11.73	19.00					
vi	6.75	12.83	16.70	125.47					
vi	11.37	19.73	19.75	19.95					
vi	20.95	20.91	20.47	19.69					
vii	8.83	4.70	5.89	4.11					
vii	6.27	9.62	5.56	3.29					
vii	7.17	14.74	19.87	18.98					
vii	7.67	10.48	4.06	4.09					
vii	8.72	10.22	10.97	5.76					
vii	11.36	6.11	3.08	3.59					
viii	16.88	23.28	23.44	23.13					
viii	14.31	21.08	22.07	25.37					
viii	12.45	13.68	17.47	11.89					
viii	23.81	25.09	32.37	30.40					
viii	23.23	19.52	24.91	22.48					
viii	4.73	14.84	28.82	22.68					
ix	5.42	3.71	2.12	1.31					

ix	14.41	20.86	23.76	31.29					
ix	4.87	13.51	8.33	3.37					
ix	13.73	20.70	20.14	22.28					
ix	8.12	1.04	5.09	2.44					
ix	18.04	24.75	24.49	24.67					
H	14.02	18.53	12.65	28.98	24.35	31.50	40.73	35.27	48.13
H	12.75	16.93	0.67	30.10	32.60	135.97	36.24	41.78	40.70
H	16.79	21.25	26.06	27.78	35.44	39.66	34.90	43.16	47.00
H	11.18	21.64	24.86	31.21	34.74	34.07	40.29	39.68	47.16

Litter Liveweight Data (g)

	d1	d2	d3	d4	d5	d6	d7	d8	d9	d10
Day 5	81.19	85.92	94.65	101.55	108.20					
Day 5	78.82	87.70	96.31	103.34	109.39					
Day 5	81.48	89.61	101.80	114.65	127.72					
Day 5	67.54	72.76	82.76	93.79	100.13					
Day 5	72.66	79.77	92.82	104.44	110.83					
Day 5	73.69	83.12	94.49	103.30	107.22					
i	88.09	101.39	110.92	116.89	123.78	124.79	128.43	129.66	133.35	131.89
i	92.16	103.35	115.94	126.96	135.24	143.83	142.28	143.60	139.82	135.66
i	77.55	90.82	100.68	109.59	115.40	120.79	125.63	131.22	138.94	145.20
i	87.52	94.34	100.16	108.86	115.49	119.32	122.56	127.06	131.53	135.62
i	83.59	95.12	103.59	117.33	125.38	128.42	130.85	134.98	136.98	140.30
i	73.43	86.01	99.05	110.33	119.99	124.65	129.48	133.63	139.18	140.35
ii	72.94	82.99	89.88	97.17	101.54	105.42	111.03	117.63	124.93	127.85
ii	77.95	91.22	102.36	111.67	123.62	126.54	132.95	141.19	148.85	150.66
ii	87.02	100.68	105.35	125.95	131.15	140.72	149.56	157.57	164.33	172.03
ii	64.94	73.55	81.96	90.72	99.83	106.65	114.80	125.41	134.90	140.38
ii	86.65	99.47	109.58	117.96	122.25	130.43	131.76	139.88	142.82	149.74
ii	80.52	92.53	102.35	109.26	116.62	123.50	127.38	128.49	131.20	133.09
ii	87.76	97.97	109.48	118.69	128.33	135.63	138.83	140.78	141.50	140.80
iii	83.61	93.90	105.44	116.38	129.25	138.30	149.26	158.50	166.48	173.75
iii	97.99	91.66	103.69	115.73	124.04	134.17	149.68	160.55	168.80	178.43
iii	65.79	76.40	90.64	103.01	117.10	131.41	143.27	156.83	168.79	177.68
iii	84.48	95.09	95.71	97.30	97.72	106.97	114.56	122.47	133.09	137.93
iii	81.53	92.90	102.80	110.78	118.37	128.86	149.72	158.39	166.83	175.33
iii	84.54	94.96	105.23	115.45	125.46	133.00	147.17	158.08	166.26	175.07
iv	74.94	86.47	97.28	107.91	114.72	126.08	137.95	151.23	160.94	173.94
iv	81.13	94.02	97.08	104.38	107.46	115.64	128.33	141.81	152.90	166.23
iv	89.99	99.06	104.11	109.63	110.70	112.48	119.41	130.03	138.17	150.24
iv	80.54	87.57	100.66	112.25	124.42	137.48	151.17	165.10	176.34	187.37
iv	79.61	89.67	95.97	104.04	111.14	119.63	131.31	142.20	150.92	161.75
iv	77.23	85.54	95.77	100.72	105.78	113.75	125.66	140.01	151.48	162.18
v	80.83	91.78	102.21	114.92	125.12	140.72	157.46	169.76	185.32	198.06
v	86.67	94.97	102.33	107.87	112.24	122.55	136.84	150.44	163.67	174.95
v	77.74	86.77	98.75	112.61	125.66	143.86	160.18	174.39	190.90	204.74
v	78.46	88.19	90.67	91.40	93.92	105.88	119.46	135.28	146.59	157.56
v	77.89	90.34	97.44	104.53	107.94	121.71	137.71	152.94	167.44	180.10
v	79.13	92.35	98.39	106.01	111.27	122.06	136.67	149.99	165.29	177.60
vi	79.30	86.12	94.53	101.92	109.33	121.11	135.82	150.90	174.20	191.05
vi	88.85	99.82	111.76	118.87	126.71	138.62	155.74	174.28	190.98	205.61
vi	90.92	96.56	104.14	113.44	120.85	137.03	153.08	170.39	186.86	198.90
vi	73.38	80.92	88.04	97.29	105.32	120.06	137.21	153.79	173.26	191.54
vi	83.99	92.66	98.53	106.99	113.17	119.62	137.89	154.30	167.35	186.39
vi	83.10	94.90	106.35	116.77	126.16	142.94	160.53	176.86	192.86	208.21
vii	84.82	97.30	105.97	113.20	117.53	134.12	155.42	179.68	201.76	220.85
vii	83.33	89.33	98.85	105.99	108.42	121.61	140.59	156.32	181.22	202.83
vii	84.91	94.39	104.02	113.66	124.12	138.02	157.56	175.92	196.52	208.08
vii	82.22	97.81	104.96	111.24	118.42	130.86	152.30	175.16	201.59	223.27
vii	73.16	80.57	90.49	95.93	101.75	120.81	144.09	162.56	186.03	210.29
vii	85.48	98.47	103.91	109.48	115.25	130.84	152.78	176.34	200.27	221.30
viii	81.18	91.93	103.61	112.88	120.18	135.12	155.67	171.91	188.95	207.08
viii	85.12	99.78	110.06	120.54	133.15	150.24	172.24	189.28	205.02	218.83
viii	83.45	94.10	102.01	110.11	117.51	131.70	148.11	163.43	178.28	192.57
viii	82.43	92.08	103.11	116.21	131.21	150.18	170.81	187.96	206.20	225.98
viii	87.67	96.85	103.41	115.92	122.85	143.09	160.04	174.42	188.18	200.73
viii	74.34	83.28	93.77	104.15	112.96	130.79	149.92	166.96	184.84	201.55
ix	88.19	100.34	104.19	107.21	110.76	125.16	141.33	159.80	181.98	213.51

ix	83.49	93.74	105.52	116.37	126.37	140.39	167.99	185.29	215.97	239.83
ix	78.10	88.59	96.90	104.82	109.71	126.20	145.22	165.80	191.76	221.25
ix	81.75	93.64	103.78	111.14	119.52	137.67	162.33	185.31	209.34	230.68
ix	62.37	66.06	74.31	78.41	80.12	90.12	102.14	119.01	136.92	163.48
ix	92.28	103.35	114.61	126.24	136.21	151.76	176.02	195.00	233.11	243.43
H	90.57	100.44	110.14	118.24	127.36	143.75	170.82	196.98	222.14	247.59
H	81.57	94.33	108.80	121.94	138.39	162.29	187.39	211.42	235.54	265.34
H	84.53	99.67	116.07	134.57	158.49	186.35	215.28	239.90	267.03	300.03
H	83.88	95.30	106.27	121.13	140.22	161.50	190.07	213.86	242.82	265.42

Mammary and Milk Composition Data

	gwt	DM	tDNA	tPro	tRNA	RD	PD	lact	prot	lipid	lactsyn
1	297.52	11.51	15.39	2.34	53.73	3.49	152.16	10.92	94.13	91.60	25.58
1	406.36	15.46	16.53	3.78	45.05	2.73	228.40	12.21	82.16	172.60	36.48
1	302.58	11.52	16.86	3.37	104.76	6.21	199.99	18.19	77.48	193.70	35.27
1	369.85	12.97	9.29	2.24	48.33	5.20	241.02	9.62	83.69	175.80	79.01
1	327.15	9.90	7.85	1.72	54.52	6.94	218.77	7.98	103.27	125.70	80.68
1	346.03	14.90	10.78	1.97	62.07	5.76	182.48	16.42	86.24	206.20	54.36
2	296.68	7.64	6.21	2.30	114.65	18.48	370.68	16.34	65.83	143.60	50.41
2	407.65	7.73	13.56	2.19	85.77	6.33	161.74	17.90	71.08	215.20	64.24
2	331.90	14.40	18.24	3.06	99.68	5.46	168.02	17.49	67.25	207.30	40.26
2	296.13	8.02	11.85	2.11	83.84	7.07	178.31	13.70	66.03	115.20	53.31
2	299.29	8.79	12.95	2.83	65.72	5.07	218.19	11.55	68.48	187.20	62.17
2	296.94	6.62	5.77	1.80	73.40	12.72	311.82	3.99	110.54	141.90	22.27
3	302.28	5.32	9.41	1.98	88.20	9.37	210.49	23.53	101.90	170.90	65.94
3	302.01	4.05	7.30	1.70	73.28	10.04	233.00	30.39	63.40	125.80	63.96
3	295.79	3.20	7.22	1.53	61.66	8.54	212.31	14.01	101.79	215.50	56.43
4	293.31	2.68	8.14	1.45	60.86	7.48	178.41	12.52	127.04	168.49	51.78
4	289.13	4.95	10.13	1.96	99.31	9.80	193.23	17.96	81.43	148.70	95.50
4	322.34	3.54	5.49	2.03	86.18	15.71	370.88	19.41	71.37	111.80	43.65
5	289.99	4.72	16.43	2.75	113.16	6.89	167.57	15.69	62.83	139.60	155.37
5	294.75	3.14	7.15	1.87	76.05	10.64	262.04	22.31	81.10	152.10	111.09
5	338.74	4.72	12.19	2.45	120.11	9.86	200.68	17.00	84.71	161.70	139.42
5	215.03	3.28	7.43	1.57	66.74	8.98	210.85	23.30	79.57	168.90	218.31
5	321.24	5.71	5.20	2.31	106.70	20.53	445.16	11.54	96.60	189.40	152.65
5	374.05	3.90	17.06	1.95	109.52	6.42	114.13	6.99	134.79	235.80	142.82
6	302.97	5.59	11.80	2.10	109.91	18.96	178.26	15.03	68.51	120.50	177.75
6	323.69	5.53	14.37	2.07	78.96	5.50	144.33	18.87	76.91	215.70	141.61
6	306.07	6.81	22.23	2.91	102.20	4.60	130.72	12.65	96.86	75.50	122.34
6	321.99	4.54	9.29	1.96	60.93	6.56	211.44	11.47	99.16	117.50	200.07
6	323.91	3.90	14.18	2.56	95.95	6.77	180.88	18.95	93.67	189.30	214.89
6	355.96	7.11	17.71	3.16	101.02	5.70	178.46	13.31	92.52	159.70	224.60
7	310.53	6.05	17.87	2.52	183.06	10.24	140.96	*	*	*	191.60
7	297.57	5.30	6.03	2.74	94.42	15.67	454.43	26.28	68.61	105.20	209.20
7	319.70	7.78	10.51	2.49	133.35	12.69	236.96	21.42	75.24	145.90	125.24
7	301.83	6.04	11.05	2.37	47.90	4.33	214.07	20.99	63.10	116.20	254.03
7	308.92	5.09	16.28	2.30	140.86	8.65	141.23	23.18	77.35	120.73	237.28
7	328.47	5.22	20.92	2.35	96.99	4.64	112.34	16.05	65.92	154.20	216.14
8	309.55	6.15	15.04	2.38	122.29	8.13	158.33	28.63	58.08	139.70	157.45
8	283.30	6.69	18.25	2.30	131.60	7.21	125.83	26.56	73.67	141.90	168.72
8	259.62	7.54	20.95	2.75	127.61	6.09	131.30	*	*	*	180.82
8	325.36	6.72	16.56	4.73	49.96	7.61	285.42	18.27	84.04	207.20	178.85
8	325.77	7.86	20.92	3.56	175.37	8.38	170.04	23.59	78.18	137.10	200.68
8	300.81	5.09	16.19	2.54	108.74	6.71	156.90	17.58	82.84	134.40	121.81
9	301.56	7.06	22.38	2.81	168.77	7.54	125.44	18.90	75.29	115.55	154.79
9	306.86	7.53	12.31	3.53	130.72	10.62	286.46	18.69	97.59	121.30	311.26
9	294.12	5.93	12.12	3.00	123.13	20.10	247.29	16.99	73.01	137.00	265.35
9	336.70	11.22	12.80	3.96	179.49	14.02	309.12	24.93	63.55	95.20	266.29
9	315.94	7.48	7.28	2.58	99.63	13.69	354.44	24.46	79.74	153.10	332.67
9	297.19	4.88	13.82	2.82	150.56	10.90	203.95	14.63	75.84	129.40	263.47

10	310.08	6.68	7.15	3.13	125.52	17.56	438.04	20.79	93.64	162.80	176.86
10	346.56	6.24	6.79	2.85	72.40	10.66	419.90	20.68	75.38	134.50	163.39
10	301.33	4.21	11.41	3.15	127.58	11.18	276.14	23.97	81.74	125.00	164.85
10	307.61	7.91	13.81	3.26	105.66	7.65	235.68	13.92	85.55	136.80	164.65
10	281.72	4.26	27.09	2.48	123.01	4.54	91.41	16.99	100.23	157.20	100.74
10	301.66	4.08	16.74	2.11	71.73	4.28	125.80	12.75	107.33	198.80	119.76
11	306.96	9.76	17.02	3.96	174.23	10.24	232.83	19.89	107.53	117.80	196.85
11	302.04	12.09	23.48	5.07	220.42	9.39	215.81	29.91	59.59	84.60	256.53
11	315.27	9.33	35.51	3.92	164.12	4.62	110.48	20.78	89.23	129.00	224.22
11	316.32	6.95	13.65	2.76	220.60	16.17	201.89	20.58	72.54	120.50	211.69
11	348.24	8.59	15.07	3.37	166.20	11.03	223.27	21.86	99.11	100.50	230.71
11	304.38	9.05	19.33	3.55	165.35	12.12	183.81	21.04	88.05	67.20	256.76
12	305.19	8.90	24.80	3.94	129.41	5.22	158.73	23.60	106.56	103.30	253.20
12	300.59	7.89	15.61	3.48	113.67	7.28	223.17	21.49	97.34	111.10	225.39
12	398.95	11.18	17.34	4.43	161.01	9.29	255.78	26.78	54.44	103.60	228.79
12	285.87	9.19	16.39	2.86	131.11	18.54	174.54	24.21	95.33	63.60	273.72

Carcass Composition Data

Trt	gwt	carDM	carCP	carF	carA	oDM	oCP	oF	oA	pDM	pCP	pA	pF
1	297.52	64.18	33.36	24.47	7.82	32.28	6.82	25.10	0.58	28.34	10.77	0.23	17.34
1	406.36	80.18	30.56	40.16	9.14	73.81	7.53	64.07	0.48	45.03	13.24	0.26	31.53
1	302.58	57.04	30.01	21.36	8.25	31.58	4.46	24.13	0.36	27.67	10.49	0.23	16.96
1	369.85	74.04	35.02	30.26	7.52	39.25	6.72	30.81	0.46	34.25	12.16	0.24	21.85
1	327.15	66.08	36.72	20.81	7.14	27.04	9.02	17.11	0.51	27.74	13.20	0.24	14.30
1	346.03	79.06	35.45	35.11	7.82	60.87	6.30	45.14	0.53	44.80	14.02	0.27	30.50
2	296.68	55.52	31.50	16.23	8.10	25.86	5.07	20.23	0.43	22.78	12.10	0.24	10.44
2	407.65	63.86	34.53	21.54	7.46	36.44	4.37	30.04	0.47	29.28	11.74	0.23	17.31
2	331.90	79.28	38.19	33.12	8.01	40.83	6.37	32.62	0.46	38.52	12.63	0.30	25.59
2	296.13	56.00	31.43	17.27	6.83	33.84	5.77	27.21	0.25	25.93	12.03	0.26	13.64
2	299.29	55.90	31.39	17.91	7.11	38.68	5.25	31.96	0.53	23.23	12.50	0.21	10.53
2	296.94	55.00	33.30	14.90	7.10	35.47	4.45	28.42	0.41	28.91	9.97	0.27	16.94
3	302.28	43.69	26.72	7.88	6.32	12.38	3.74	7.54	0.99	12.92	10.50	0.23	2.19
3	302.01	42.22	29.26	7.32	6.68	17.99	4.32	11.45	0.50	15.74	10.61	0.21	4.92
3	295.79	45.91	27.73	11.24	6.66	14.29	2.92	10.41	0.26	15.96	10.60	0.19	5.18
4	293.31	37.74	26.82	5.17	7.48	12.64	3.39	7.63	0.22	12.95	10.26	0.19	2.51
4	289.13	43.42	25.63	11.12	7.13	32.31	4.12	27.11	0.23	20.95	11.90	0.23	8.82
4	322.34	45.20	31.39	7.66	7.14	16.95	3.95	12.44	0.37	15.46	8.26	0.22	6.98
5	289.99	42.53	27.82	8.07	7.36	16.16	3.35	11.84	0.33	15.73	9.97	0.24	5.52
5	294.75	46.56	28.13	11.06	7.04	15.23	3.50	10.59	0.40	16.72	11.52	0.26	4.94
5	338.74	47.66	31.03	9.33	7.42	21.95	4.15	16.03	0.62	16.93	11.75	0.24	4.94
5	215.03	40.90	26.25	8.13	7.68	13.99	3.46	9.54	0.39	14.67	10.50	0.20	3.96
5	321.24	49.71	31.40	13.77	6.95	17.71	3.70	13.96	0.44	19.73	11.44	0.24	8.04
5	374.05	56.15	35.59	14.84	6.86	21.23	3.60	18.29	0.27	19.81	12.60	0.23	6.98
6	302.97	53.63	30.84	15.51	7.12	20.29	3.49	15.85	0.41	20.10	11.56	0.37	8.17
6	323.69	54.39	33.69	13.68	7.76	20.51	3.83	14.65	0.34	19.29	13.14	0.22	5.93
6	306.07	41.40	25.23	8.94	7.48	25.01	4.18	19.21	0.49	16.84	10.86	0.20	5.77
6	321.99	57.49	33.48	16.24	6.96	19.95	4.30	15.07	0.39	24.34	11.42	0.27	12.66
6	323.91	44.93	31.16	9.43	7.72	18.36	3.78	13.01	0.42	15.52	11.36	0.20	3.95
6	355.96	49.26	32.22	9.95	7.67	22.06	3.26	17.53	0.30	21.45	11.90	0.27	9.28
7	310.53	47.16	27.73	12.58	7.71	20.61	4.60	14.53	0.45	16.66	12.08	0.21	4.37
7	297.57	49.86	32.59	9.46	6.86	23.61	4.83	18.44	0.30	24.73	11.23	0.23	13.27
7	319.70	43.69	23.81	14.91	7.61	27.05	4.85	21.01	0.33	16.68	11.68	0.21	4.79
7	301.83	58.63	36.09	13.68	5.84	23.50	5.12	17.57	0.46	18.27	11.05	0.20	7.02
7	308.92	40.70	25.09	8.36	9.23	23.98	4.52	18.63	0.23	17.04	10.28	0.23	6.54
7	328.47	44.64	34.04	7.27	6.65	20.95	4.14	16.03	0.33	18.08	11.95	0.21	5.92
8	309.55	48.26	27.64	13.34	6.09	31.99	5.20	25.51	0.43	19.33	11.68	0.20	7.46
8	283.30	52.58	28.19	17.09	6.80	21.82	5.59	16.04	0.38	18.48	11.18	0.22	7.08
8	259.62	52.37	27.93	17.65	7.31	23.15	4.73	17.96	0.43	20.22	9.04	0.22	10.96
8	325.36	49.98	24.38	19.58	7.06	21.94	4.75	16.05	0.48	19.30	10.04	0.22	9.05
8	325.77	55.63	34.22	13.39	6.17	23.28	4.10	18.33	0.42	21.32	11.70	0.18	9.43
8	300.81	52.90	32.49	14.44	8.01	21.53	3.83	16.85	0.47	16.84	9.87	0.20	6.77
9	301.56	45.87	29.36	9.85	7.46	25.32	4.95	17.81	0.31	18.90	10.45	0.23	8.21
9	306.86	51.01	29.08	14.75	6.75	23.55	6.06	16.06	0.81	17.06	11.84	0.32	4.90
9	294.12	45.38	31.50	7.79	7.35	18.86	5.58	12.92	0.46	17.24	11.10	0.23	5.91
9	336.70	56.49	29.63	20.47	6.77	39.59	4.96	32.98	0.29	26.02	11.71	0.27	14.05
9	315.94	55.68	31.04	17.02	7.31	23.48	4.87	17.09	0.45	22.40	10.91	1.44	10.05
9	297.19	45.98	28.62	10.76	7.18	14.53	4.80	8.99	0.07	15.28	10.60	0.23	4.45

10	310.08	61.44	38.24	17.78	7.08	27.38	5.08	19.14	1.14	20.78	11.18	0.22	9.38
10	346.56	52.38	34.05	10.75	7.27	22.14	4.25	16.06	0.45	20.12	11.63	0.27	8.22
10	301.33	41.96	32.23	4.66	7.80	12.14	3.98	6.88	0.76	12.54	9.46	0.19	2.89
10	307.61	64.67	32.62	24.77	6.63	23.29	4.87	17.63	0.27	23.89	9.13	0.25	14.51
10	281.72	39.11	27.36	5.83	7.52	10.03	3.36	5.44	1.47	13.73	10.65	0.26	2.82
10	301.66	47.26	33.30	7.52	6.51	13.03	4.21	8.08	0.71	15.14	10.90	0.24	4.00
11	306.96	52.36	31.54	13.89	7.49	29.77	6.71	21.97	0.27	20.71	10.71	0.26	9.74
11	302.04	64.09	28.42	27.84	7.12	22.95	4.59	17.06	0.55	26.04	11.64	0.26	14.14
11	315.27	51.54	35.30	11.37	6.93	32.64	5.78	25.73	0.32	21.65	11.82	0.25	9.58
11	316.32	49.99	32.75	10.31	7.15	22.26	4.99	16.80	0.28	18.16	11.31	0.24	6.60
11	348.24	55.12	32.54	14.69	7.28	27.47	5.90	20.38	0.42	20.85	11.70	0.24	8.92
11	304.38	49.56	34.57	8.09	7.69	22.83	6.08	16.34	0.21	17.71	10.77	0.27	6.68
12	305.19	50.02	32.35	10.49	7.36	26.22	5.17	19.59	0.27	19.33	9.63	0.25	9.45
12	300.59	47.05	32.80	7.54	7.77	20.99	6.37	14.48	1.02	18.67	10.92	0.27	7.48
12	398.95	66.35	38.50	18.61	6.98	31.91	6.12	24.09	0.49	22.93	12.66	0.27	10.01
12	285.87	53.39	30.56	15.02	7.89	22.52	7.58	13.94	0.82	21.21	11.84	0.22	9.16

Genstat5 (ver.3.1) Programs for Trial 3

Live Animal - Analysis of Covariance

```
"Trial 3,  Lactational Measurements - Oct.'95 (January 1996)"
"Trt - Treatment
 gwt - dam body weight at day 1 gestation
 damga1 - dam lactation weight gain,  days 1 to 5
 damga2 -                                6 to 10
 dmi1 - dam dry matter intake,        days 1 to 5
 litga1 - litter weight gains, mean   days 1 to 5
 litga2 -                                6 to 10"
units [nvalues=52]
factor [levels=10; labels=!t(i,ii,iii,iv,v,vi,vii,viii,ix,H)]Trt
output [width=80]1
read [channel=2]Trt, gwt,damga1, damga2,  dmi1, dmi2, litga1, litga2

treat Trt
" Analysis of covariance for dam wt change over first five days with gwt as only
covariate"
covariates gwt

anova [fprob=yes; print=aovtable,covariates, means]damga1, damga2,dmi1, dmi2, \
litga1, litga2, cpi, ncpge
```

Mammary and Milk Composition - Analysis of Covariance and Multivariate Regression Analysis

```
"Trial 3 - Oct.'95 (February 1996)"
"Trt - Treatment
 gwt - day 1 gestation weight
 CPI - acumulated crude protein intake (g)          days 1 - 5
 NCPGE - non crude protein gross energy intake (kJ) days 1 - 5
 DM -  Total mammary dry matter (g)
 tDNA - Total mammary DNA (mg)
 tPro -  Total mammary protein (g)
 tRNA - Total mammary RNA (mg)
 RD - RNA:DNA ratio
 pro - Milk Protein (mg / g milk)
 lact - Milk Lactose
 lipid - Milk Lipid
 lactsyn - lactose synthetase activity (nmoles lactose / mg DNA / min)
"
units [nvalues=64]
factor [levels=12; labels=!t(Day1, Day5,i,ii,iii,iv,v,vi,vii,viii,ix,H)]Trt
read [channel=2]Trt, gwt, DM, tDNA, tPro, tRNA, RD, lact, pro, lipid, lactsyn

treat Trt
"Analysis of covariance with day 1 gestation wt as only covariate"
covariates gwt
anova [fprob=yes; print=aovtable, covariates, means]mDM, tDNA, tPro, tRNA, \
RD, lact, pro, lipid, lactsyn

"Multivariate regression analysis"
model DM, tDNA, tPro, tRNA, RD, lact, pro, lipid, lactsyn
calc int=(CPI-mean(CPI))*(NCPGE-mean(NCPGE))
fit [tprob=yes; fprob=yes; print=sum,cor,acc,est,mod] gwt,CPI,NCPGE,int
model DM, tDNA, tPro, tRNA, RD, lact, pro, lipid, lactsyn
fit [tprob=yes; fprob=yes; print=sum,cor,acc,est,mod] gwt,NCPGE, CPI, int
```

Carcass Composition - Analysis of Covariance and Multivariate Regression Analysis

```
"Trial 3 - Oct.'95 (July  1996)"
"Trt - Treatment gwt - day 1 gestation weight
 CPI - acumulated crude protein intake (g)          days 1 - 5
 NCPGE - non crude protein gross energy intake (kJ) days 1 - 5
 cardm - carcass dry matter (g)
 carf - total carcass fat (g)
 carcp- total carcass crude protein (g)
 cara- carcass ash(g)
 odm - organs dry matter (g)
 otcp - total organ crude protein (g)
 of - total organ fat(g)
 oa - total organ ash (g)
 pdm - pelt dry matter (g)
 pcp - pelt total crude protein (g)
 pash - pelt total ash (g)
 pfat - estimated total pelt fat (g)
 cof - carcass plus organ fat (g)
 cocp - carcass plus organ crude protein (g)
 cptcp - carcass+pelt total crude protein (g)
 cpocp - carcass + organ + pelt total fat (g)
"
close channel=2
open 'carcass1.gdt'; channel=2; width=300
output [width=80]1
units [nvalues=64]factor [levels=12;
labels=!t(day1,day5,i,ii,iii,iv,v,vi,vii,viii,ix,H)]Trt
read [channel=2]Trt, gwt, cardm, carcp, carf, cara, odm, ocp, of, oa, pdm, pcp, \
pa, pf, cocp, cofat, copcp, copfat
treat Trt

" Analysis of variance - day 1 gestation BW covariate"
covariate gwt
anova [fprob=yes; print=aovtable, covariate, means]cardm, carcp,carf,cara, odm, ocp,\
of, oa, pdm, pcp, pa, pf, cocp, cofat, copcp, copfat

calc int=(cpi-mean(cpi))*(ncpge-mean(ncpge))
model cptcp, cardm, carcp, carf, cara, odm, ocp, of, oa, pdm, pcp, \
pa, pf, cocp, cofat, copcp, copfat
fit [tprob=yes; fprob=yes; print=acc,sum,mod,corr,est] gwt,cpi,ncpge,int

"Multivariate regression analysis"
model cardm, carcp, carf, cara, odm, ocp, of, oa, pdm, pcp, \
pa, pf, cocp, cofat, copcp, copfat
fit [fprob=yes; tprob=yes; print=acc,sum,mod,corr,est] gwt,ncpge,cpi,int
```

A2.5 DATA FOR TRIAL 4

Maternal Liveweight Data (g)

	d1	d2	d3	d4	d5	d6	d7
HH	403.96	391.49	396.85	396.51	400.59	396.96	403.83
HH	393.80	388.85	386.90	385.41	386.95	381.13	381.95
HH	338.63	334.28	324.43	326.75	325.58	322.22	320.86
HH	467.18	470.47	467.80	465.43	463.34	461.51	460.91
HH	300.99	292.25	293.27	299.90	302.08	307.12	310.20
HH	422.28	414.51	418.08	406.75	405.21	408.70	412.55
HH	424.15	429.02	412.44	411.14	424.90	410.74	405.26
HH	371.36	367.40	365.47	370.40	368.71	370.66	364.15
LH	381.42	371.96	370.15	358.61	347.52	335.44	362.31
LH	368.62	366.29	358.62	349.48	342.29	336.24	369.79
LH	351.56	337.71	321.23	313.04	302.02	289.83	304.74
LH	393.27	377.91	362.75	354.66	339.37	326.28	349.99
LH	401.35	397.77	398.08	380.40	363.93	357.95	375.05
LH	420.17	418.58	408.47	401.54	395.40	373.52	387.31
LH	403.38	395.62	393.92	393.04	373.28	356.22	367.86
LH	394.83	383.24	369.66	356.34	349.98	335.65	340.57
LL	408.76	386.77	380.89	379.95	368.34	355.64	348.50
LL	416.65	400.10	389.43	376.32	353.08	343.74	331.60
LL	368.62	366.29	358.62	349.48	342.29	336.24	319.69
LL	427.78	421.32	408.91	399.16	380.74	365.11	356.38
LL	395.05	385.15	382.19	364.13	353.05	333.71	320.45

Maternal Food Intake Data (g DM)

	d2	d3	d4	d5	d6	d7
HH	11.88	23.71	29.23	31.11	38.00	41.96
HH	11.77	13.95	24.84	24.98	23.23	28.25
HH	13.64	16.54	20.56	26.66	25.79	28.38
HH	19.48	22.22	28.35	29.79	30.49	37.33
HH	11.38	26.37	31.98	33.30	38.90	39.48
HH	13.78	17.85	18.68	25.11	30.84	35.02
HH	19.65	10.85	24.99	31.85	31.55	33.85
HH	16.10	20.69	30.89	37.71	37.71	32.72
LH	12.15	17.25	14.83	19.56	9.07	38.22
LH	9.98	9.69	7.01	2.77	0.26	21.84
LH	5.55	3.49	3.27	2.78	2.12	19.34
LH	5.19	6.34	2.07	3.27	3.35	25.67
LH	15.55	24.51	22.28	7.12	18.95	35.60
LH	16.10	20.66	24.28	17.47	3.45	31.46
LH	12.36	11.77	19.99	7.95	3.91	26.33
LH	11.67	10.61	6.43	4.67	2.59	17.31
LL	5.12	14.94	19.44	17.44	9.01	9.28
LL	9.29	17.69	15.80	4.25	1.97	2.16
LL	17.04	16.36	20.82	20.87	16.81	4.05
LL	17.87	13.68	14.10	5.58	0.99	3.26
LL	9.46	9.14	9.55	6.85	5.96	3.12

Litter Liveweight Data (g)

	d2	d3	d4	d5	d6	d7	d8
HH	86.78	103.10	118.47	141.45	164.85	191.51	217.50
HH	80.47	93.33	103.12	115.21	133.35	151.11	171.04
HH	94.21	108.36	121.89	138.70	156.44	170.65	190.06
HH	85.49	100.45	115.18	134.81	157.07	178.31	200.28
HH	77.41	89.64	106.95	128.43	152.00	178.40	203.78
HH	77.02	78.78	88.30	103.20	120.12	140.67	165.66
HH	97.56	112.35	128.28	144.58	159.54	191.18	217.59
HH	120.44	130.15	144.47	157.93	178.99	197.24	222.20
LH	88.49	101.32	109.63	117.14	126.16	132.16	147.63
LH	79.15	94.33	101.49	109.17	112.24	112.45	127.36
LH	80.99	90.16	96.60	98.61	102.09	104.12	113.93
LH	79.20	90.78	94.49	98.54	104.28	104.60	115.21
LH	85.02	102.59	117.56	134.18	139.27	146.41	168.10
LH	94.69	106.56	120.75	128.98	136.14	139.36	160.21
LH	78.41	88.30	98.48	110.63	117.43	122.32	139.23
LH	71.79	85.21	97.21	101.30	104.24	107.66	114.24
LL	74.07	81.93	93.86	103.71	110.56	116.73	120.95
LL	85.74	92.14	110.35	118.01	120.98	121.92	126.52
LL	85.85	99.89	113.09	125.91	134.95	140.61	144.56
LL	90.52	97.52	106.58	115.37	118.48	118.71	121.76
LL	87.72	91.77	98.20	112.06	117.93	118.63	119.66

Liquid Scintillation Data

Rat	Trt	Diet	Time	d.p.m	d.p.m./h
1	1	1	1	2253.93	2253.93
1	1	1	2	4886.96	2443.48
1	1	1	3	6811.69	2270.56
1	2	1	1	1280.63	1280.63
1	2	1	2	2436.22	1218.11
1	2	1	3	3770.95	1256.98
1	3	1	1	2890.14	2890.14
1	3	1	2	5758.91	2879.46
1	3	1	3	6801.69	2267.23
2	1	1	1	1272.02	1272.02
2	1	1	2	1579.93	789.965
2	1	1	3	3529.24	1176.41
2	2	1	1	1248.19	1248.19
2	2	1	2	3890.25	1945.13
2	2	1	3	6374.93	2124.98
2	3	1	1	1745.18	1745.18
2	3	1	2	3889.76	1944.88
2	3	1	3	4742.8	1580.93
3	1	1	1	2039.25	2039.25
3	1	1	2	3591.83	1795.92
3	1	1	3	6059.83	2019.94
3	2	1	1	2638.16	2638.16
3	2	1	2	5773.73	2886.87
3	2	1	3	10234.7	3411.58
3	3	1	1	1163.26	1163.26
3	3	1	2	3511.99	1756
3	3	1	3	8217.19	2739.06
4	1	2	1	1415.93	1415.93
4	1	2	2	1767.98	883.99
4	1	2	3	4245.22	1415.07
4	2	2	1	3217.27	3217.27
4	2	2	2	12991.9	6495.95
4	2	2	3	27097.3	9032.42
4	3	2	1	1712.82	1712.82
4	3	2	2	9589.33	4794.67
4	3	2	3	26193.9	8731.28
5	1	2	1	763.27	763.27
5	1	2	2	1800.47	900.235
5	1	2	3	2522.86	840.953
5	2	2	1	3338.62	3338.62
5	2	2	2	11983.6	5991.8
5	2	2	3	23405.9	7801.95
5	3	2	1	1102.82	1102.82
5	3	2	2	14365.4	7182.7
5	3	2	3	30930.7	10310.2
6	1	2	1	1414.06	1414.06
6	1	2	2	2870.38	1435.19
6	1	2	3	5697.39	1899.13
6	2	2	1	3390.04	3390.04
6	2	2	2	10550.8	5275.41
6	2	2	3	33459.4	11153.1
6	3	2	1	1335.41	1335.41
6	3	2	2	10118.2	5059.1
6	3	2	3	25116.7	8372.22

Histological Data - Cells Undergoing Pyknosis per 10 Alveoli

Diet	Diet	Rat	Pyknosis
HH	1	1	0
HH	1	1	22
HH	1	1	21
HH	1	1	0
HH	1	1	51
HH	1	1	0
HH	1	1	10
HH	1	1	0
HH	1	1	2
HH	1	1	0
HH	1	1	0
HH	1	1	43
HH	1	1	5
HH	1	1	3
HH	1	1	0
HH	1	1	20
HH	1	2	0
HH	1	2	10
HH	1	2	0
HH	1	2	0
HH	1	2	0
HH	1	2	33
HH	1	2	0
HH	1	2	0
HH	1	2	20
HH	1	2	11
HH	1	2	9
HH	1	2	0
HH	1	2	13
HH	1	2	14
HH	1	2	0
HH	1	3	0
HH	1	3	22
HH	1	3	1
HH	1	3	27
HH	1	3	1
HH	1	3	13
HH	1	3	12
HH	1	3	8
HH	1	3	7
HH	1	3	1
HH	1	3	22
HH	1	3	0
HH	1	3	13
HH	1	3	14
HH	1	3	23
HH	1	3	0
HH	1	4	0
HH	1	4	27
HH	1	4	0
HH	1	4	1
HH	1	4	18
HH	1	4	40
HH	1	4	18
HH	1	4	0
HH	1	4	0

HH	1	4	0
HH	1	4	3
HH	1	4	2
HH	1	4	7
HH	1	4	9
HH	1	4	19
HH	1	4	7
HH	1	4	0
HH	1	4	4
HH	1	4	8
HH	1	5	7
HH	1	5	0
HH	1	5	0
HH	1	5	0
HH	1	5	0
HH	1	5	1
HH	1	5	10
HH	1	5	18
HH	1	5	20
HH	1	5	43
HH	1	5	7
HH	1	5	0
HH	1	5	14
HH	1	5	19
HH	1	5	7
HH	1	5	8
LL	2	6	4
LL	2	6	53
LL	2	6	52
LL	2	6	6
LL	2	6	25
LL	2	6	37
LL	2	6	37
LL	2	6	25
LL	2	6	16
LL	2	6	46
LL	2	6	24
LL	2	6	28
LL	2	6	39
LL	2	6	2
LL	2	6	25
LL	2	6	26
LL	2	7	28
LL	2	7	14
LL	2	7	54
LL	2	7	61
LL	2	7	29
LL	2	7	25
LL	2	7	35
LL	2	7	22
LL	2	7	2
LL	2	7	5
LL	2	7	8
LL	2	7	24
LL	2	7	15
LL	2	7	19
LL	2	7	25
LL	2	7	6
LL	2	8	24

LL	2	8	35
LL	2	8	38
LL	2	8	54
LL	2	8	46
LL	2	8	37
LL	2	8	55
LL	2	8	28
LL	2	8	8
LL	2	8	52
LL	2	8	28
LL	2	8	15
LL	2	8	13
LL	2	8	25
LL	2	8	24
LL	2	8	35
LL	2	9	6
LL	2	9	8
LL	2	9	18
LL	2	9	39
LL	2	9	57
LL	2	9	48
LL	2	9	62
LL	2	9	25
LL	2	9	26
LL	2	9	39
LL	2	9	42
LL	2	9	9
LL	2	9	14
LL	2	9	7
LL	2	9	48
LL	2	9	24
LL	2	10	57
LL	2	10	2
LL	2	10	0
LL	2	10	57
LL	2	10	38
LL	2	10	15
LL	2	10	24
LL	2	10	25
LL	2	10	5
LL	2	10	6
LL	2	10	18
LL	2	10	19
LL	2	10	38
LL	2	10	51
LL	2	10	36
LL	2	10	68
LH	3	11	13
LH	3	11	4
LH	3	11	5
LH	3	11	14
LH	3	11	0
LH	3	11	8
LH	3	11	14
LH	3	11	21
LH	3	11	18
LH	3	11	9
LH	3	11	0
LH	3	11	0

LH	3	11	4
LH	3	11	7
LH	3	11	8
LH	3	11	18
LH	3	12	29
LH	3	12	18
LH	3	12	24
LH	3	12	35
LH	3	12	17
LH	3	12	2
LH	3	12	0
LH	3	12	5
LH	3	12	19
LH	3	12	31
LH	3	12	19
LH	3	12	24
LH	3	12	15
LH	3	12	5
LH	3	12	4
LH	3	12	18
LH	3	13	28
LH	3	13	14
LH	3	13	15
LH	3	13	0
LH	3	13	2
LH	3	13	0
LH	3	13	5
LH	3	13	6
LH	3	13	24
LH	3	13	15
LH	3	13	7
LH	3	13	14
LH	3	13	21
LH	3	13	15
LH	3	13	24
LH	3	13	18
LH	3	14	17
LH	3	14	16
LH	3	14	5
LH	3	14	8
LH	3	14	14
LH	3	14	0
LH	3	14	0
LH	3	14	15
LH	3	14	24
LH	3	14	18
LH	3	14	11
LH	3	14	5
LH	3	14	16
LH	3	14	24
LH	3	14	0
LH	3	14	14
LH	3	15	8
LH	3	15	14
LH	3	15	8
LH	3	15	5
LH	3	15	22
LH	3	15	5
LH	3	15	0

LH	3	15	0
LH	3	15	0
LH	3	15	9
LH	3	15	15
LH	3	15	24
LH	3	15	14
LH	3	15	5
LH	3	15	0
LH	3	15	0

Genstat5 (ver.3.1) Programs for Trial 4

Live Animal - Analysis of Covariance

```
"Trial 4,  Lactational Measurements - August 1996"
"Trt - Treatment
 gwt - dam body weight at day 1 gestation
 damga1 - dam lactation weight gain, days 1 to 6
 damga2 -                              6 to 7
 damga3 -                              1 to 7
 dmi1 - dam dry matter intake, days 1 to 6
 dmi2 - . . . . . . . . . . . . . . . 6 to 7
 dmi3 -                             1 to 7
 litga1 - litter weight gains, mean days 1 to 6
 litga2 -                              6 to 7
 litga3 -                              1 to 7
"
units [nvalues=]
factor [levels=3; labels=!t(HH, LH, LL)]Trt
"open 'lact.dat'; ch=2"output [width=80]1
read [channel=2]Trt, gwt, damga1, damga2,  dmi1, dmi2, litga1, litga2, damga3,\
 dmi3, litga3

treat Trt" Analysis of covariance with day 1 gestation weight as covariate"
covariate gwt
anova [fprob=yes; print=aovtable,covariates,means]damga1, damga2, damga3, \
dmi1, dmi2, dmi3, litga1, litga2, litga3
```

Analysis of Cellular Proliferation Data

```
"Trial 4 - Analysis of Scintillation counting for final protocol"
"rat  -  rat useddiet -  1 is HH      and      2 is LH
 media-  1 is D-PBS;    2 is Medium 199;    3 is Medium 199 + 20% Serum
 time -  1 is 1 hour culture;   2 is 2 h culture and 3 is 4 h
 count-  is scintillation count (dpm/ug DNA)
 SA   -  specific activity (counts/ug DNA/h)
"
output [width=80]1
units [nvalues=54]
factor [levels=6]rat
factor [level=2; labels=!t(HH, LH)]diet
factor [level=3; labels=!t(PBS, OneNine, Serum)]media
factor [level=3; labels=!t(One, Two, Four)]time
factor [level=6; labels=!t(HHPBS, HHonenine, HHserum, LHPBS, LHonenine,\ Lhserum)]trt
read [channel=2]rat, media, diet, time, count, SA, trt

"Regression Testing"
block rat
treat diet*media*POL(time;1)
anova [fprob=y; print=aovtable, means, contrasts]count

"Effects of diet, media and time on total [3H] uptake"
block rat
treat diet*media*time
anova [fprob=y; print=aovtable, means, contrasts]count

"Effects of diet and media on Specific Uptake of [3H]"
block rat
treat diet*media
anova [fprob=y; print=aovtable, means]SA"

Effects of diet, media and time on Specific Uptake [3H]"
block rat
treat diet*media*time
anova [fprob=y; print=aovtable, means]SA

"Regression Analysis for Activity against Time"
treat trt
model count
fit [print=acc,mod,est,summ;fprob=y; tprob=y]time
```

APPENDIX III

PUBLICATIONS

Goodwill,M.G., Jessop,N.S. and Oldham,J.D. (1996). Mammary sensitivity to protein restriction and re-alimentation. *Protein Metabolism and Nutrition. Proceedings of the 7th International Symposium. EAAP Publication No. 81.* pp 419-420 [A.F. Nunes, A.V. Portugal, J.P. Costa and J.R. Ribeiro, editors] Santarem: EZN.

Goodwill,M.G., Jessop,N.S. and Oldham,J.D. (1996). Mammary sensitivity to protein restriction and re-alimentation. *British Journal of Nutrition.* **76.** 423-434.

Goodwill,M.G., Jessop,N.S. and Oldham,J.D. (1996). Does the histamine receptor antagonist, cyproheptadine increase voluntary food intake? *Proceedings of the Nutrition Society.* (In Press).

Goodwill,M.G., Jessop,N.S. and Oldham,J.D. (1996). Mammary Sensitivity to protein and energy intakes during lactation. *The American Dairy Science Association.* (In Press).

Goodwill,M.G., Jessop,N.S. and Oldham,J.D. (1996). Protein-energy malnutrition: responses to the histamine receptor antagonist Cyproheptadine during growth or lactation. (Submitted to the *British Journal of Nutrition*).

Goodwill,M.G., Jessop,N.S. and Oldham,J.D. (1996). Mammary sensitivity to protein and energy intakes during lactation. (To be submitted to *Journal of Nutrition*).

Goodwill,M.G., Jessop,N.S. and Oldham,J.D. (1996). Sensitivity of body tissues to protein and energy intakes during lactation. (To be submitted to *Journal of Nutrition*).